Intestinal immunology in man

Kenneth Andrew Humphreys

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Declaration

I declare that this thesis has been composed by me, and that the work contained within it, except on the occasions that were clearly stated, was performed by me

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Publications based on this thesis

Low whole gut lavage complement C3 predicts poor outcome in inactive Crohn's disease

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This abstract is included at the back of this thesis with the permission of the co-authors.

Abbreviations

Systèms international d'unités (SI) units have been used throughout. Other abbreviations include:

Ab	antibody
Ag	antigen
α1AT	alpha-one anti-trypsin
α4β7	integrin alpha 4 beta 7
α chain	heavy chain of immunoglobulin A
B-cell	B lymphocyte
BCIP/NBT	5-Bromo-4-Chloro-3-Indolyl Phosphate /Nitro Blue Tetrazolium
$CaCl_2.2H_2O$	calcium chloride
C1-C9	complement factors 1-9
C3b, C3c etc.	activation fragment produced by cleavage of native complement component
CD	Crohn's disease
CDAI	Crohn's disease activity index
CDi	inactive Crohn's disease
CDa	active Crohn's disease (i and a also used for ulcerative colits, UC)
CDAI	Crohn's disease activity index
CV	coefficient of variation
$d.H_2O$	distilled water (glass distilled)
ELISA	enzyme linked immunosorbant assay
factors B, H, I and P:	serum proteins involved in complement activation or metabolism
EDTA	ethylene diamine tetracetic acid
Fc	fraction cystalizable of immunoglobulin molecule (= N-terminal end)
g	acceleration due to gravity (10 ms^{-2})
γ chain	heavy chain of immunoglobulin G
ICAM	intercellular adhesion molecule
IgA	immunoglobulin A
IgA1 etc.,	immunoglobulin A subclass 1
IgG	immunoglobulin G
IgG1 etc.,	immunoglobulin G subclass 1 etc.
IGF-1	insulin-like growth factor 1
IL-1, IL-8 etc.	interleukin type 1, interleukin type 8 etc.
IL-1β	interleukin-1-beta (secreted form of IL-1)
IgM	immunoglobulin M
Kd	kilodaltons
$\log_{10}$	logarithm to base 10
LPMNC	lamina propria mononuclear cells
LPS	lipopolysaccharide
μ chain	heavy chain of immunoglobulin M

Abbreviations continued

mA	milliamps
MAdCAM-1	mucosal addressin type adhesion molecule one
$MgCl_2.6H_2O$	magnesium chloride
mRNA	messenger RNA
mins	minutes
MW	molecular weight
NF-Kβ	nuclear factor kappa B
ND	not done
NS	not significant
OD	optical density
P	probability of observation being random
PBS	phosphate buffered saline
PEG	polyethylene glycol
PMSF	phenyl methyl sulphonyl fluoride
QC	quality control
r	correlation coefficient
r^2	coefficient of determination for a regression line
RCE	relative coefficient of excretion for protein loss to the gut = (concentration in WGLF/(concentration in serum)/ratio for albumin
Rf	relative fractionation = distance moved by protein band/ distance moved by buffer
RNA	ribonuceic acid
rpm	revolutions per minute
rt	room temperature (20-25°C)
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate treated proteins separated by polyacrylamide gel electrophoresis
TBS	Tris buffered saline
T-cells	T lymphocytes
T-Gel	thiophilic absorption ligand coupled to Sepharose 4B
TNF-α	Tumour necrosis factor-alpha
Tris	tris(hydroxymethyl) amino methane
Tween 20	polyoxyethylen-sorbitan monolaurate
UC	ulcerative colitis
v/v	ratio of volume to volume
WGL	whole gut lavage (procedure)
WGLF	whole gut lavage fluid
w/v	ratio of weight to volume
χ^2	chi squared, statistic for comparing proportions
x	times (multiply)
yrs	years

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ABSTRACT

Direct investigation of the intestinal immune system is difficult since tissues are relatively inaccessible. Faeces is a readily available source of intestinal contents which may contain proteins and other substances secreted from the mucosa. However, protein recovery is affected by gut proteases and differences in whole gut transit time, limiting the value of any conclusions.

The potential of whole gut lavage fluid (WGLF) as a source of intestinal proteins has been evaluated. The clear effluent obtained following routine bowel cleansing with a polyethylene glycol based isotonic solution can be rapidly processed with the addition of protease inhibitors, preventing further proteolytic digestion. The gut lavage procedure reduces and standardizes the transit time, allowing comparison of protein secretion.

Detection of antigen specific antibodies by capture enzyme linked immunosorbant assay (ELISA) requires an intact antigen binding site to be associated with heavy chain. As protein degradation in WGLF is less than for faeces, antibody detection should be possible. Proteolysis of WGLF immunoglobulins, especially those lost from more proximal regions of the gut, could lead to underestimation of intestinal humoral immunity. The first aim of this thesis was to validate WGLF as a source of intact intestinal antibodies.

Up to 85% of WGLF immunoglobulins had bound to a thiophilic ligand (T-Gel) which has been shown to specifically absorb intact immunoglobulins from biological fluids. Subsequent to SDS-PAGE and western blotting, anti heavy chain staining of the 55 Kd heavy chain band was more intense with T-Gel binding as opposed to unfractionated immunoglobulins. There was also greater recovery of antigen specific antibodies in the T-Gel binding fraction. These findings may indicate that WGLF immunoglobulins are largely intact and that estimation by ELISA was likely to be quantitative.

Loss of IgG to the gut in patients with inflammatory bowel disease (IBD) can arise from plasma or by local production from cells in the lamina propria.

The source of IgG in WGLF was shown to be predominantly plasma since the relative coefficient of excretion for IgG in WGLF was similar to albumin for all IBD patients. To limit the effect of greater susceptibitity to proteolysis of albumin compared to other WGLF proteins, the study was restricted to patients with colonic disease. Secondly, only WGLF without high protease activity was used. It has been assumed that protein loss occurs from the site diagnosed as diseased by clinical assessment.

To confirm that plasma leakage was the major source of WGLF IgG in patients with colonic IBD, loss of another plasma protein, complement C3, was investigated. A competitive ELISA was developed to quantify C3 in WGLF. This required binding of a single antibody enabling detection of both immune complexes and free C3. WGLF C3 was raised in patients with active IBD. This loss mirrored that of IgG and albumin, showing no diagnostic specificity. In patients with ulcerative colitis, the relative loss of C3 was similar to that for albumin. In contrast, patients with colonic Crohn's disease (CD) had twofold loss of C3 relative to albumin, indicating that mucosal production was a major contributor to WGLF C3. As patients with inactive CD had slightly raised WGLF C3, this may be an indicator of early clinical relapse.

The relationship between WGLF C3 and relapse of CD was investigated by follow up of 43 patients for one year after gut lavage. Of 10 who relapsed, 8 had normal WGLF C3 whereas of 15 patients who remained in steroid free remission, 8 had raised WGLF C3. There was a risk factor of 4.4 for relapse in patients with inactive CD who had normal WGLF C3. This suggested either complement deposition or a protective role of complement e.g., in clearing apoptotic cells. These hypotheses could be further investigated using intestinal biopsies.

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Background and aims

Direct investigation of immune activity within the intestinal mucosa is difficult because of the inaccessibility of the tissue. Indirect means of study include collection of substances secreted into the gut lumen e.g., by use of a perfusion system. Research at the G.I unit, Edinburgh has shown that routine bowel preparation by whole gut lavage can be easily adapted to give reproducible bowel perfusion. Collection of the effluent perfusion fluid per rectum and subsequent addition of protease inhibitors has provided a useful medium in which to quantify substances secreted from the intestinal wall (O'Mahoney et al, 1991).

Previous work from the department has shown that some proteins e.g., IgA are detectable in WGLF from all subjects. This is likely to reflect physiological secretion. Only low concentrations of other proteins such as IgG, albumin, IL-1 and IL-8 were detected in WGLF from most subjects but concentrations were increased in patients with active inflammatory bowel disease (IBD). Clinical studies have demonstrated selectivity of intestinal loss of these proteins (Arnott et al, 1997).

Values of WGLF IgG above the upper 95th percentile of that observed in a non-inflammatory population have been shown to correlate with clinical indices of disease activity disease (the Powell-Tuck index for ulcerative colitis and the Crohn's disease activity index) in patients with IBD (Choudari et al, 1993). Subjects with IBD showing intestinal symptoms may undergo the WGLF procedure in preparation for further intestinal examination. This made it relatively easy to collect specimens and quantify IgG as a guide of the severity of intestinal inflammation.

In contrast, calculation of clinical indices has been relatively complicated, requiring clinical assessment over seven days in the case of the CDAI. Consequently, it has been tempting to use biochemical markers of inflammation to reflect disease activity. Before adapting this approach, one should understand that this is an isolated measure of one component of disease activity i.e. loss of a particular protein across the gut wall. With a damaged mucosa, that protein may come from plasma or local production as part of the local inflammatory event.

The WGLF concentration of that protein might therefore overestimate the mucosal inflammation component of disease activity.

The inactive phase of IBD is a state of stable chronic inflammation characterized by greater numbers of B and T-cells in the intestinal mucosa than seen in healthy individuals (Rosekrans et al, 1980). These cells may be in a quiescent state and produce low concentrations of inflammatory mediators. These may be undetectable in WGLF, either because of the limitations of assay sensitivity or because the intestinal epithelium is impermeable to them. Changes in the permeability of the intestinal epithelium might allow movement of proteins from the mucosa to the gut lumen and visa-versa. Movement of antigenic proteins from the gut lumen to the intestinal mucosa may provide the stimulus for activation of the mucosal immune compartment (Gardiner et al, 1995).

The active phase of disease is an acute inflammatory reaction exacerbated by a hyper-reactivity of the mucosal immune system. The early phase is characterized by increased migration of neutrophils into the intestinal mucosa.This has been demonstrated by detection of neutrophil proteins such as elastase and calprotectin in the gut lumen (Tibble et al, 2000). Subsequently, B and T lymphocytes are activated resulting in exacerbation of the acute inflammatory reaction. Proteins such as IgG may then be detectable in the intestinal contents. Biochemical markers of the early phase of the acute inflammatory response in IBD may provide a useful indication of the need for clinical intervention before full relapse occurs.

Despite published work on WGLF, the technique is yet to be validated in terms of analyte recovery. Some proteins e.g., albumin are particularly susceptible to proteolysis. Further work has been necessary to evaluate differences in protein recovery as a prelude to considering relative loss of each protein. This could then be exploited in consideration of the source of proteins detected in WGLF. The first aim of this thesis was to evaluate the integrity of proteins in WGLF and how this affected their detection. Because of the use of IgG as a marker of inflammation, it was decided to focus on immunoglobulins.

Following validation of protein recovery in WGLF, other proteins which might reflect intestinal inflammation could be investigated. Complement protein C3 might be involved in exacerbation of the intestinal inflammatory reaction via IgG mediated activation of the complement cascade. Alternatively, C3 might also be activated by the alternative pathway via interaction with luminal bacteria. To investigate the role of C3 in intestinal inflammation it was decided to establish a method for quantifying C3 in WGLF.

The remainder of the thesis has focused on clinical studies to evaluate the relationship between detection of C3 in WGLF and intestinal inflammation. This incorporates work to elucidate whether the C3 lost to the gut comes from serum or might be produced in the intestinal mucosa. Finally, the potential for WGLF C3 to predict relapse of Crohn's disease has be studied. This work has focused on Crohn's disease partly because of interesting preliminary findings with WGLF C3. Furthermore, CD is a heterogeneous condition with increasing incidence which has high morbidity amongst young people (Armitage et al, 1999). Management of CD has been more problematic than that of UC, in which surgery is an effective method.

AIMS

1) To test whether proteins in WGLF are intact or not, using immunoglobulins as an example. Chromatography will be used to extract immunoglobulins from the protein mixture before subjecting the immunoglobulins to SDS-PAGE electrophoresis to assess their molecular size and the likely extent of proteolysis. The relevence of these results to the reliability of quantifying proteins in WGLF will be discussed.
2) To develop an ELISA for quantification of complement component C3 in WGLF.
3) To compare WGLF C3 in patients with and without intestinal inflammation to see if this parameter is an indicator of the severity of inflammation.
4) To use albumin as a marker of plasma leakage to identify whether C3 lost to the gut is likely to come from plasma or might be synthesized by the intestinal wall.
5) To investigate the relationship between WGLF C3 and disease outcome in patients with inactive Crohn's disease. A biochemical indicator of the likely course of disease would be a useful tool to the gastro-intestinal physician.

CHAPTER ONE

Review of the literature

1.1 Study of the intestinal immune system

The gastro-intestinal immune system is physically inaccessible, creating practical and ethical difficulties to its study in man. The intestinal wall is difficult to access and material can be obtained only in requirement for histological investigation of a clinical condition. For research purposes, biological fluids would be easier to obtain. Serum might be expected to contain some of the substances produced in the gut wall. However, it may not be valid to extrapolate detection of a protein in serum to immunological activity in the gut wall: patients with ankylosing spondylitis or Crohn's disease had raised serum IgA to the gut bacterium Klebsiella. However, only the CD patients show raised specific IgA in WGLF. (O'Mahoney et al, 1992). Immunological function of the intestinal mucosa should therefore be investigated using material obtained from the gut.

1.2 Intestinal disorders where study of mucosal immunity may be useful

1.2.1 Inflammatory bowel disease

Ulcerative colitis and Crohn's disease and are chronic, spontaneously relapsing disorders of the intestine. Although the cause is unknown, they are immunologically mediated and have genetic and environmental influences. Symptoms for the two diseases are very varied and diagnosis is made on the basis of histological examination of inflamed intestine. Differences in the type and site of inflammation in the two diseases may indicate different causes. Many of the secondary processes which exacerbate inflammation are common to both diseases. It would be of interest to know which agents the intestinal immune response in IBD is directed against and why there are abnormal reactions to this agent.

Immunological mediators produced during active disease may provide clues to the nature of the inflammatory pathways, so contributing to the development of new treatments. Biological markers of intestinal inflammation may also provide a tool for monitoring response to treatment or prediction of clinical outcome.

1.2.2 Coeliac disease

Coeliac disease is an intolerance of the intestinal immune system to dietary gluten. Ingestion of gluten causes a powerful immune response characterized by increased numbers of intraepithelial lymphocyte, especially of those bearing $\gamma\delta$ receptors (Arranz et al, 1994). In addition, there is also abnormal intestinal production of IgA antibodies to gliadin, a digestion product of gluten. New evidence suggests that these antibodies are produced as a by product of an antibody response to an enzyme, tissue transglutaminase, which is involved in protein cross-linking (Dieterich et al, 1997). Gliadin is a substrate for this enzyme and the gliadin-transglutaminase complex may reveal novel epitopes against which an immune response may be mounted (in subjects having the necessary major histocompatability antigens/ T-cell receptor). Transglutaminase is involved in tissue re-modeling and is probably only released in response to tissue injury. Patients with untreated coeliac disease show a flat intestinal mucosa (no villi) with crypt hyperplasia and rapid loss of immature epithelial cells to the gut. On removal of gluten from the diet, a complete recovery is seen. This may indicate that tissue transglutaminase is required in the healing process. The intestinal antibody response to gliadin or transglutaminase could be monitored using WGL, providing an indicator of compliance to the gluten free diet.

1.3 Invasive methods of obtaining intestinal material

Material can be obtained from the human intestine by either invasive or non-invasive methods. Invasive methods often include those for clinical investigation of a particular patient. It may be difficult to obtain suitable patient groups and controls. Intestinal examination by endoscopy involves insertion of a flexible tube fitted with a camera into the intestinal lumen. Forceps attached to the endoscopy tube allow the taking of a mucosal biopsy. Intestinal secretions can be aspirated via a draining tube..

1.3.1 Intestinal biopsy

Intestinal biopsy specimens have been used in several ways to study gut physiology. Once the tissue has been treated with preservatives and thin sections cut, the fixed image of the mucosa can be viewed by microscopy. Cells of a particular type are recognized either by their morphology or by staining of cell surface components with specific antibodies.

Individual cell types can be isolated and grown in culture. This allows investigation of cell specific changes e.g. in response to inflammatory mediators. Some cell types e.g., epithelial cells are difficult to grow, possibly because they lack interactions with other cell types that are essential for regulation of their growth. Immortalized cell lines have been used as models of intestinal cell types. However, cell lines tend to be at an earlier differentiation stage and do not behave like differentiated intestinal cells. For example, the intestinal epithelium-like cell, Caco-2 produces IL-1 and IL-6 in culture but freshly isolated intestinal epithelial cells do not (Daig et al, 2000). Organ culture may be more physiological but it may be impossible to identify the functions of individual cell types..

Other problems with the study intestinal biopsy specimens include the requirement to strictly control for the anatomical site so that site specific differences in tissue physiology are not assumed to be disease specific. When looking at changes in disease, specimens from several sites along the intestine should be taken. This would avoid bias due to a particular specimen showing few or extensive signs of inflammation.

Control material for these studies has been typically taken from the edge of tumour resections. Despite a histologically normal appearance, this material may have had biochemical abnormalities.

1.3.2 Intestinal secretions

Jejunal fluid can be collected into a draining tube at endoscopy. As jejunal fluid tends to be rich in pancreatic proteases, the recovery of secreted proteins may be low. Also, secretion is not over a fixed time period and this limits the conclusions that can be drawn from it's analysis. Jejunal fluid is therefore of little use for monitoring subtle changes in mucosal immune activity but may allow detection of gross changes e.g. detection of antigliadin IgA in coeliac disease (Volta et al, 1988).

The intestinal wall can be 'washed' at endoscopy and these washings will contain secreted proteins. This may flush out pre-formed proteins and so not reflect protein synthesis at that time. As only a small area of the intestinal mucosa is studied, abnormalities elsewhere may be missed..

1.4 Non-invasive methods of obtaining intestinal material

1.4.1 Faeces

Faeces is simple to collect and contains proteins secreted from the whole gut . However, measurement of proteins from faeces has several drawbacks. In addition to proteins secreted from the intestinal mucosa, faeces contains bacteria, digestive enzymes, dead cells and food breakdown products. These have been shown to interfere with quantification of faecal proteins by ELISA (Viscidi et al, 1984).

Protease enzymes either of host or gut bacterial origin are capable of degrading proteins within faeces (Macfarlane et al, 1986). The extent of protein degradation will depend on the properties of the protein (Lebek and Cottier 1992), the activity and type of enzymes present and the time that the protein is in contact with those enzymes.

Diarrhoeal stools may take less time to pass through the colon than a solid stool and so this material may have been subjected gut proteases for less time. Protein recovery might therefore be greater from diarrheoal stools than from solid stools. It is difficult to say that greater protein concentrations of diarrhoeal stools only reflect increased secretion in that disease. We have demonstrated that specific IgA to food antigens was detectable more frequently in diarrhoeal stools than in solid stools. This was not seen in WGLF from the same patients. Immunological tests on faeces may therefore be misleading (Ferguson et al, 1995a).

Protein recovery from faeces may depend on the chemical nature and extraction efficiency of that protein e.g. IgA has been shown to be bound to bacterial antigens and these may sediment upon centrifugation (Retz and Steele 1977). The water content of the stool will influence the protein concentration and so must be taken into account before comparisons can be made. As the production of faeces is variable, the quantity produced per unit time needs to be known before protein secretion can be compared (Meillet et al, 1987).

1.4.2 Gut Perfusion

A non absorbable fluid was used to wash the intestinal walls, collecting any released proteins. These studies are usually performed in the fasting state to reduce the degradation of collected proteins by digestive enzymes and contamination with food or faeces. A perfusion is established when the rate of fluid influx into the intestine balances the rate of fluid collection. Intestinal perfusion allows study of protein secretion over a known time period.

Polyethylene glycol (PEG) in a balanced salt solution has been used for intestinal perfusion. It is chemically inert, not absorbed by the gut and the salt content prevents net water movement (Beck et al, 1985).

PEG is a large water soluble molecule which mixes well in aqueous solutions. It can be assayed chemically or labelled with radioactive markers. Complete recovery of PEG has been demonstrated following transit through healthy or inflamed colon (Soergel, 1968).

Although PEG is chemically inert, it does interact with proteins (Busby and Ingham, 1980) and it can interfere with immunoassays, particularly by disrupting antigen binding to plastic surfaces (Tobi et al, 1991)

1.4.2.1 Closed loop perfusion systems

These are a specialized means of collecting proteins secreted from a particular gut segment. An inflatable balloon is used to isolate a segment of the intestine and a system of tubes allows separate fluid infusion and collection (Knutson et al, 1989). The placement of segmental perfusion apparatus is difficult and uncomfortable for the patient.

An advantage of perfusing an occluded gut segment is that secreted proteins are uncontaminated by fluid from the upper bowel (which may include pancreatic enzymes). This fluid is aspirated clear of the balloon and a dye can be used to show that leakage into the perfused segment does not occur (Rambaud et al, 1981). It has been suggested that this technique is not detrimental to mucosal integrity, e.g., stretching of the bowel by the balloon does not alter protein secretion compared to that seen without a balloon in patients with pancreatic insufficiency (Rambaud et al, 1981). However, extended use of an occluding balloon may be detrimental to mucosal integrity as 3/9 healthy volunteers showed increased secretion of complement and albumin on the second day (Hoj et al, 1981).

Effective perfusion requires efficient mixing of the luminal contents. This might not occur when the bowel is fully distended using rapid flow rates of greater than 7 ml/min (Wingate et al, 1974). In healthy volunteers, immunoglobulin secretion was unaltered by changing the perfusion rate from 5 to 10 ml/min (Brown et al, 1988). The use of a completely closed loop improves the recovery of perfusate. A lower perfusion rate can then be used and secreted proteins are diluted less (Knutson et al, 1989).

One drawback of segmental perfusion is that in study of a defined area, abnormalities elsewhere in the intestine are not detected. Crohn's disease is characterised by discontinuous inflammation along the length of the intestine. The site and volume of bowel perfused must be taken into account before comparing protein secretion in different patients (Soergel, 1971).

Perfusion of the intestine with PEG solution is artificial in terms of the antigen load to the gut and greater protein secretion is seen using a nutrient perfusate (Colombel et al, 1992). Drinking large volumes of solution might alter gut physiology by activating intestinal hormone and neuronal systems (Shah et al, 1982). This might stimulate protein secretion.

1.4.2.2 Whole gut lavage

At the Gastro-Intestinal Laboratory (Western General Hospital, Edinburgh), whole bowel perfusion with polyethylene glycol solution 'klean-Prep'(Norgine, Oxford, UK), has been used to investigate patients undergoing examination for gastro-intestinal disease. Intestinal secretions can be obtained routinely from per-oral polyethylene glycol based bowel cleansing (gut lavage) prior to colonoscopy or barium enema (Gaspari et al, 1988). This technique of bowel cleansing is well tolerated by most patients (Ernstoff et al, 1983). Standardization of the intestinal secretions requires the patient to drink at a standard, consistent rate until the rectal effluent is clear of faecal matter.

Once the fluid passing per rectum was clear, the rate of drinking has been shown to equal the rate of excretion i.e. a steady state perfusion system (Choudari et al, 1993). It has been demonstrated that protein secretion into the gut during the whole gut lavage procedure was at a steady state. Protein concentrations were equivalent in five sequential liquid stools, collected after passing of the first clear specimen (Sallam,J. Ph.D. thesis, University of Edinburgh 1993).

The whole gut lavage fluid has been processed by filtration and addition of protease inhibitors to prevent further proteolysis of proteins (Brydon et al, 1993). It has been shown that immunuglobulins in processed WGLF were stable for up to two years when stored frozen at -70°C (Åhrén et al, 1995).

Advantages of WGLF

The main advantages of the WGL technique as opposed to other forms of intestinal perfusion are that it is easy to perform and material from patients is readily available. A technical advantage is that the whole bowel is perfused.

Disadvantages of WGLF

1. The main disadvantage of studying proteins in WGLF is that there is no way of knowing which region of the intestine those proteins are secreted from. It is assumed that inflammatory mediators are secreted from regions of the intestine where there is radiological and endoscopic evidence of inflammation.
2. Although the effect of gut proteases is reduced because of the short transit time, some proteolysis of secreted proteins is likely to occur. This might have a greater impact on proteins secreted more proximally in the gut.
3. As high flow rates of 17 ml/min are required to establish a perfusion, the secreted proteins are diluted, limiting their detection.

Disadvantages common to all sources of intestinal secretions

1. Analysis of a single specimen may give limited information if there was intermittent protein loss, for example, blood loss to the gut has been shown to be intermittent (Bouma et al, 1996).
2. It is impossible to control for plasma, lymph or interstitial fluid contents leaking into the gut. This limits the conclusions that may be drawn from detection of a protein. The source of proteins leaking into the gut has been studied using radiolabelled proteins (Jarnum and Jensen, 1975) or, by comparison with albumin, a marker of plasma leakage (Jonard et al, 1984).

1.4.2.3 Results of studies using WGLF

Leakage of plasma proteins to the gut resulting from intestinal inflammation

In patients with active IBD, intestinal loss of albumin, α1AT, and IgG has been shown to correlate with the disease activity as assessed by clinical indices based on both subjective and objective measures (Choudari et al, 1993). Measurement of these proteins in WGLF therefore provides a simple way assessing whether disease is active and can be used to monitor changes is disease activity. In some circumstances, e.g., in patients with Crohn's disease where the CDAI is heavily weighted by subjective factors, measuring WGLF IgG may provide a more accurate indicator of mucosal inflammation (Acciuffi et al, 1996). The presence of these proteins in WGLF shows high but not absolute specificity for IBD as raised levels are seen in many other conditions, including colorectal cancer (Brydon et al, 1993).

The mechanism of inflammation in IBD

Inflammatory cellular migration into the intestinal mucosa has been studied using WGLF. Neutrophils have been counted by cytology (Handy et al, 1995) or indirectly by measuring granulocyte elastase (Handy et al, 1996). Numbers of neutrophils in WGLF were increased in patients with active IBD, particularly colonic disease. This supports a theory that neutrophil migration may be enhanced in response to chemotactic factors from luminal bacteria, these being more numerous in the colon. Neutrophils are activated by chemotactic signals or by uptake of foreign material. This results in release of cytotoxic granules containing powerful enzymes and production of reactive oxygen metabolites. These anti-bacterial functions have the potential to cause bystander damage to the intestinal mucosa.

Changes to intestinal humoral immunity in inflammatory gut disorders

The intestinal immune system usually functions in a state termed oral tolerance. This is actually a misnomer which represents a specialized response against luminal contents in which mainly IgA is produced and secreted across the gut wall. The significance of this is that some antigens are excluded from the intestinal mucosa and this avoids inflammatory reactions by immune cells in the intestine. Systemic immune responses to immunological challenge elsewhere is down-regulated (oral tolerance reviewed by Weiner, 1997).

Alterations in intestinal humoral immunity could include changes in the specificity of IgA produced, or a switch to production of IgG. This immunoglobulin is less well adapted to antigen exclusion and more likely to trigger an inflammatory reaction. Immunological changes in the gut have been detected by quantifying immunoglobulins in WGLF. Patients with either coeliac disease or IBD had WGLF IgA specific for food proteins more frequently than did healthy controls. In addition, these patients also had serum IgG specific for food antigen whuch may have indicated greater permeability of the intestinal wall to luminal antigen (Ferguson et al, 1996).

In another study, WGLF IgA specific for the outer coat of Bacteroides was quantified by ELISA. WGLF from patients with CD gave significantly greater OD readings than those with other gastrointestinal disorders or healthy controls (Poxton et al, 1995). However, as samples with high readings in the plateau region of the standard curve were not re-tested at greater dilution, some results may have been underestimated. This may have contributed to the finding that IgA specific for Bacteriodes did not correlate with total IgA. However, other studies of intestinal humoral immunity also suggest poor correlation of specific and total IgA because the specific IgA only contributes up to 5 % of the total.

Production of cytokines by the inflamed intestine

Children with cystic fibrosis had detectable WGLF levels of pro-inflammatory cytokines IL-1β and IL-8, measured using commercial ELISA kits. Children who were taking high dose enzyme supplements had high levels of WGLF cytokines and so may have been at risk of intestinal inflammatory damage (Croft et al, 1996).

These cytokines have a short half life in serum and so are likely to have resulted from immune activity within the mucosa. Quantification of cytokines in WGLF can provide mechanistic information about the nature of intestinal inflammation.

Patients with active IBD also had raised WGLF IL-1 and IL-8. Unexpectedly, patients with inactive CD also had slightly raised WGLF cytokines, indicating that remission of CD may be a state of low grade intestinal inflammation (Arnott et al, 1997). When patients with inactive CD were stratified into low and high WGLF cytokine groups, more of the patients in the high cytokine group were shown to relapse during a one year follow-up period (Arnott et al, 1998). This finding might have implications for patient management.

Healing of inflamed intestine

The healing process in IBD may be studied by measuring extracellular matrix components such as hyluronic acid or enzymes involved in tissue remodeling, e.g. metalloproteinases. Healing of intestinal lessions may in itself produce more problems such as narrowing of the intestinal lumen by fibrotic strictures in CD. Fibrosis involves the production of excess collagen during tissue remodelling. The possible role of hormones which control collagen synthesis e.g. insulin like growth factor (IGF-1) and transforming growth factor beta (TGF-β) have been investigated by measuring these in WGLF. This demonstrated that IGF-1 was detectable most frequently in WGLF from CD patients with fibrotic strictures (Ghosh et al, 1997).

Monitoring of patient response to therapy

Loss to the gut of plasma proteins (as indirect markers of inflammation) or cytokines (as markers of intestinal immune activation) may provide a means of demonstrating improvement of intestinal inflammation in response to therapy. Patients with active IBD show elevated WGLF albumin and IL-1β. Following treatment with elemental diet, these WGLF parameters returned to normal levels (Ferguson et al, 1998). WGLF IgG has been used as a tool for stratifying patients in clinical trials (Ferguson et al, 1998).

Use of WGLF as a research tool by other workers

Other research groups have used the technique of WGLF for obtaining intestinal material. In Italy, IgG1 and IL-1 were quantified in WGLF from children to assess intestinal inflammation (Troncone et al, 1997). A group in Holland has detected eicosanoids in WGLF and related these to the severity of mucosal inflammation (Hommes et al, 1996). And in Japan mucin has been extracted from WGLF and chemical differences in mucin from patients with IBD investigated (Saitoh et al, 1996).

1.5 Problems of quantifying proteins in biological fluids

When using antisera to detect proteins, it is important to consider the specificity of antisera used because biological fluids may contain a plethora of potentially cross reactive epitopes. This problem is compounded in WGLF where the action of protease enzymes could create new, potentially cross reactive epitopes. PEG might also interfere in assays by promoting antibody-antigen associations (Lachmann, 1991).

1.5.1 The effect of gut proteases on protein recovery

Secretory IgA in colostrum has been shown to be more resistant to digestion by trypsin or duodenal fluid than are serum immunoglobulins (Brown et al, 1970). After digestion of colostrum with duodenal fluid for 8 h at 37°C, 57 % of the original α chain activity was detectable. Recovery of serum IgA and IgG was much lower. Digest proteins were fractionated using gel filtration and the fractions assayed by radial immunodiffusion with anti heavy chain. In the colostrum digest, 51 % of the original α chain activity resided in the fraction corresponding to intact sIgA with only 10 % being detected in low molecular weight fractions. It was therefore unlikely that an assay for IgA based on binding of anti α chain antisera would overestimate IgA in the digest because of detection of small fragments with α chain determinants.

In another study, human sIgA was shown to be more resistant to digestion with pancreatic elastase. The size of digest products was analysed by ultracentrifugal analysis. IgG and monomeric IgA were digested to give Fab and Fc fragments whereas secretory IgA was resistant to digestion (Tax and Korngold, 1971).

Nick Croft at the Gastro-Intestinal Laboratory has shown that 22% of IgA in WGLF is recovered after incubating unprocessed WGLF at 37°C for 8 h. There was little inter-patient variation in IgA recovery as quantified by ELISA for α chain (Ph.D thesis, University of Edinburgh 1996). Digestion of secretory IgA in WGLF is therefore more rapid than was observed using purified pancreatic enzymes or duodenal juice. The reason for this may be because in addition to pancreatic proteases, WGLF may contain bacterial proteases from the colon. The catalytic rate of these proteases does depend on the protein substrate (Macfarlane et al, 1986).

The extent of protein degradation in WGLF will depend on gut protease activity and the time that proteins remain in contact with the proteases. This second factor depends on the region of the intestine from which proteins are secreted and the gut transit time. The gut transit time for whole gut lavage has been measured in a preliminary study with six patients (Jamal Sallam, Ph.D thesis, University of Edinburgh, 1993). This was assessed by comparing the volume of 'Klean-Prep' drunk per unit time with the volume of WGLF excreted. This gave a plot of two graphs which were roughly parallel, demonstrating that a perfusion had been established. The time lapse between the two lines equates to the gut transit time; this varied from one hour for an ileostomy patient to three hours for a patient with constipation i.e., much shorter than for faeces. These studies are difficult for the patient as they must continue drinking the Klean-Prep solution beyond the time required to produce a clear specimen to enable calculation of fluid excretion per unit time. The estimation of transit time might have been simplified by inclusion of a dye in the ingested solution.

As the total transit time for WGLF is about 2 h, the time for proteins secreted into the intestine to reach the rectum will be shorter than this. Proteolysis of secreted proteins may be less than expected.

The proteolytic activity of WGLF can be estimated by dye release from Azocoll. This reagent consists of collagen beads which are peptide linked to a red dye. Proteases break the peptide link and release the dye, the quantity of colour of which is measured spectrophotometrically. Protease activity as measured by this method is completely inhibited by the addition of protease inhibitors (Gaspari et al, 1988). The effect of WGLF proteases on recovery of a specific protein can be examined by spiking unprocessed WGLF with a known quantity of that protein. At set timepoints, aliquots of the WGLF are taken and protease inhibitors added. The relationship between protein recovery and time in contact with WGLF proteases can then be examined. It may be useful to look at protein recovery during gut transit by inclusion of an internal protein standard such as ovalbumin in the ingested solution. The likely extent of protein proteolysis during gut transit might be indirectly estimated by WGLF protease activity.

1.5.2 Implications of gut protease action for quantification of WGLF proteins

Less than expected detection of WGLF immunoglobulins may indicate proteolysis. However, it is not known whether detected immunoglobulin represents intact molecules or fragments. Detection of fragments might result in immunoglobulin overestimatation. Despite the finding that immunoglobulin fragments were not detected disproportionately using a radial immunodiffusion assay, this may not be true for more sensitive ELISA methods. ELISA to detect antigen specific antibodies may not detect immunoglobulin fragments as an intact antigen binding site must be associated with heavy chain. Proteolysis might lead to underestimation of antigen specific antibodies.

1.5.3 Relationship between immunoglobulin structure and stability in WGLF

IgA

Most intestinal IgA is produced as a dimer of two monomer IgA units linked with a short joining chain between their Fc regions. Immunoglobulin producing cells in the intestinal lamina propria show upregulated expression of joining chain, which increases the proportion of dimeric IgA produced. Joining chain has affinity for the poly-immunoglobulin receptor located on the baso-lateral surface of intestinal enterocytes. This directs transport of IgA produced in the lamina propria to the intestinal epithelium. On binding to this receptor, dimer IgA is internalized within the enterocyte and becomes associated with a glycoprotein portion of the receptor which has been enzymatically cleaved (Brandtzaeg, 1978). This portion is termed the secretory component and it wraps around the secreted dimer IgA. This makes the immunoglobulin chains less accessible to intestinal proteases, improving the stability of secretory IgA (Underdown and Dorrington 1974).

Different IgA subclasses show different resistance to proteolytic digestion, e.g., IgA1 is cleaved in the flexible hinge region by proteases from Haemphilus (Ahl and Reinholdt, 1991) . Binding of Fab fragments can mask bacterial epitopes without opsonization of the bacteria, potentially enabling them to avoid detection by the immune system (Russell et al, 1989). IgA2 lacks this hinge region and so is more resistant to proteolytic digestion. The proportion of cells producing IgA2 as opposed to IgA1 increases from the ileum to colon. This may be an adaptation to the increased numbers of bacterial in the colon.

Monomeric IgA is susceptible to proteolytic digestion as is not associated with joining chain or secretory component and is predominantly IgA1. Most monomeric IgA is from serum.

IgM

Intestinal IgM is pentameric, associated with joining chain, and is non-covalently associated with secretory component. In intestinal secretions, IgM and secretory component readily dissociate (Hjelt et al, 1988) and this may make IgM more susceptible to proteolytic digestion.

IgG

IgG is not associated with secretory component and would be expected to show similar stability to IgM. There may be differences in the stability of the IgG subclasses e.g. IgG3 has fifteen inter chain disulphide bonds which may prevent the molecule from fragmenting even if cleavage at some points in the chains had occurred.

The extent of proteolysis of immunoglobulins in intestinal secretions may be revealed by structural analysis, allowing the potential impact on methods for their quantification to be assessed.

1.6 Methods for study of the structural nature of immunoglobulins

Conventional methods for the purification of immunoglobulins, such as affinity chromatography with anti immunoglobulin heavy chain ligands, may be of use as a preliminary procedure but can not fractionate intact from fragmented molecules. However, methods for separation of proteins on the basis of size do not work with solutions containing a high concentration of PEG such as WGLF. This is because interactions between PEG and protein result in the apparent molecular size being much larger than expected (Skoog, 1980). This is probably because the hydroxyl residues in PEG can form hydrogen bonds with peptide bonds or the amino acid side chains. An extraction technique not based on molecular size is required.

A method has been described which preferentially binds intact immunoglobulins of different classes (Hutchens and Porath, 1986). This method employs a novel absorption ligand which comprises a thio-ether group showing thiophilic affinity for immunoglobulins (called a T-Gel).

The method has been optimized to show high specificity for immunoglobulins and was subsequently been used to purify immunoglobulins from human colostrum (Hutchens et al, 1989). In this study the eluted immunoglobulins were shown to be intact by a combination of gel filtration and SDS-PAGE. This approach has potential for separating intact and fragmented immunoglobulins from WGLF.

To verify this finding with WGLF immunoglobulins, two different methods were used. As fragmented immunoglobulins are not detected by ELISA for antigen specific antibodies, intact immunoglobulins should give a higher ratio of specific to total immunoglobulins. Comparing this ratio in T-Gel binding and non-binding fractions should clarify whether WGLF immunoglobulins are intact or not. Secondly, immunoglobulins can be separated using polyacrylamide gel electrophoresis (SDS-PAGE). After transfer of the separated proteins to a blot, immunoglobulin heavy chain could be detected by probing with anti heavy chain antisera. Fragmented immunoglobulins might show detection of heavy chain at several molecular weights.

1.6.1 SDS-PAGE

Theory

Proteins are electrically charged and so will be attracted to an opposite electrical charge. If an electrical field is applied, proteins will migrate towards the positively charged anode or negatively charged cathode, depending on its net charge. This movement is called electrophoresis. The direction of this movement is dependent on the chemical nature of the protein i.e. the amino acid side groups and also the pH of the solution, which affects the charge on those side groups. The chemical nature of different proteins allows a mixture of proteins to be separated on the basis of their charge.

A supporting medium is required within which proteins can migrate but do not flow non-specifically. Gels with a lattice like structure containing pores work best. Polyacrylamide is now the most commonly used medium because a gel of predictable and uniform pore size can be produced by using a particular concentration of acrylamide. Also, polymerized acrylamide is relatively inert and does not bind to proteins.

The migration of large proteins is restricted by the pore size of the gel. Electrophoretic migration of a protein therefore depends both on charge and size. Separation of proteins solely on the basis of their size requires treatment to make differences in charge obsolete. Treatment with Sodium lauryl sulphate adds plenty of negatively charged groups to any protein, swamping its natural charge. This treatment also encourages protein unfolding because interactions between charged amino acids on different parts of the protein are disrupted. Unfolded proteins have a similar shape and so migration is dependent on their molecular weight.

Eelectrophoresis performed in a single gel can give poor resolution of dilute protein solutions because some proteins are separated by the time that others enter the gel. This problem has been resolved by employing a two part gel; the first or stacking gel is large pore and encourages rapid migration. The second or resolving gel is of a smaller pore size which retards the rapidly migrated proteins. Protein is concentrated against this boundary between the gels, so creating an even starting point. The use of two buffers, a lower pH for the stacking gel and a higher pH for the resolving gel also encourages this, ensuring that there are sharp bands for resolved proteins of a particular molecular weight (Laemmli, 1970)

1.6.1.1 Western blotting

The position of separated proteins in polyacrylamide gels can be demonstrated using a chemical stain. Unfortunately, direct identification of separated proteins is not possible as the pore size of the gel is too small to allow free diffusion of the antibodies. Separated proteins must first be transferred to a medium which will support antibody binding. This is done by 'blotting' the separated proteins onto a membrane such as nitrocellulose which is 'sticky' for proteins. An electric field is used to promote protein migration from the gel and onto the membrane, giving the same pattern of separation as on the gel (Reviewed by Towbin and Staehelin, 1989).

1.7 What is the source of proteins lost to the gut ?

Proteins in the gut lumen include those of the commensal gut bacteria and those lost from the intestinal wall. Bacteria can interfere with detection of host proteins e.g., immunoglobulins bind to bacterial cell walls. In addition, bacteria secrete proteases that may degrade secreted proteins.

Host proteins in the gut might include those from serum and those produced within the intestinal mucosa. It is difficult to tell which source a secreted protein is from but the following approaches have been used:

1. Intravenous injection of radiolabelled proteins with detection of the label in the gut (faeces).
2. Use of a serum protein as a biochemical marker of plasma leakage.

Both have their benefits and problems.

1.7.1 Radioactive markers

Radioactivity is a safety hazard, both to the patient and to the investigator. An apparent advantage of using a radiolabelled marker is that it is stable and so is likely to be detectable in the gut even if the protein carrier is degraded. However, in a study monitoring faecal excretion of intravascular injected radiolabelled albumin, relatively lower excretion was noted in patients with proximal as compared with distal Crohn's disease. As excretion of radiolabelled IgG was similar in both cases, this indicated that albumin which is excreted more proximally may be degraded to a greater degree and that the radiolabelled peptides might be reabsorbed by the intestine (Jarnum and Jensen, 1975).

1.7.2 The use of albumin as a marker of plasma leakage

Albumin may be a more specific marker of plasma leakage to the gut as compared with other plasma proteins. It is not synthesized in the intestine, it has a relatively long half life in serum (Rothschild et al, 1973) and there is no specific transport mechanism for movement of albumin from serum or extravascular spaces to the gut.

In healthy individuals only small quantities of albumin are lost to the gut. In inflammation, there may be an increased exchange of vascular and extravascular albumin (Ballmer et al, 1994). If permeability of the intestine i.e. of the basement membrane and epithelial cell junctions is increased, then increased loss of albumin to the gut is likely.

One pitfall of using albumin as a marker of plasma leakage is that it is susceptible to proteolytic digestion in the gut. Albumin lost from the ileum is likely to be degraded to a greater degree than that from the colon. This is supported by the finding that albumin in faeces was low in patients with active CD but high in patients with active ulcerative colitis (UC) (Saitoh et al, 1995). Contrary to this, WGLF albumin was high for both diseases (Choudari et al, 1993). This discrepancy could be because the gut transit time for WGLF is much shorter than for faeces.

Other serum proteins have been used as markers of serum leakage e.g. faecal clearance of alpha-1-antitrypsin (α1AT) is increased in patients with IBD (Kapel and Gobert, 1992). The volume of plasma lost per day has been calculated from the quantity of α1AT lost per day/ serum α1AT concentration (Saitoh et al, 1995).

α1AT is highly resistant to proteolytic digestion and so might show more reliable recovery in faeces or WGLF. α1AT might therefore be a very useful marker of plasma leakage. However, there is evidence that cells resident in the intestinal mucosa may produce α1AT e.g. monocytes (Van Furth et al, 1983) or intestinal epithelial cells (Molmenti et al, 1993). This might be more so in response to inflammatory stimuli. It may be useful to quantify production of α1AT in the intestinal mucosa e.g., by using culture of biopsy material and assay of the culture supernatant.

1.7.3 Relative coefficient of excretion for protein secretion to the gut

Protein secretion into the jejunum was studied using an occluding balloon perfusion system in subjects without intestinal immune abnormalities (Jonard et al, 1984). As the perfused intestinal segment is occluded from contact with intestinal proteases, secreted proteins were not digested.

The following proteins; orosmucoid (a serum protein), albumin and immunoglobulins pIgA, mIgA, IgM and IgG were quantified both in the intestinal perfusate and in serum. The perfusate protein concentration was compared to the serum concentration and this ratio expressed relative to that observed for albumin (which is serum derived).This gave the relative coefficient of excretion (RCE) i.e.

(concentration of protein in perfusate/ concentration in serum)/(ratio for albumin)

By definition, the RCE for albumin is 1.0 and so an RCE for the other proteins greater than this indicated either a specific transport mechanism or synthesis of that protein within the intestinal mucosa.

The use of albumin as a marker of passive plasma leakage was validated by measuring the recovery of an intravenous injection of radiolabelled albumin in healthy subjects. The specific activity (ratio of labeled to unlabelled) of albumin was similar in perfusate and serum, indicating that all the albumin was from serum. In contrast, the specific activity of perfusate pIgA was much lower than that in serum, indicating that most of the secreted IgA was produced in the intestinal mucosa.

Using the RCE, polymeric IgA and IgM had RCE values greater than 1.0. This reflects intestinal synthesis of these proteins and polyimmunoglobulin receptor mediated transport across the gut epithelium. Other proteins such as IgG and orosmucoid had RCE values less than one, indicating that these were serum derived. The RCE values for these proteins was inversely proportional to the molecular weight of the protein, indicating that leakage of these proteins from serum was restricted and dependent on their size.

1.7.3.1 Use of the RCE in inflammation

Non-specific movement of proteins from blood vessels into tissues may be via gaps between overlapping vascular endothelial cells. Normally these gaps are small and restrict protein movement according to their size. However, in inflammation these gaps are enlarged resulting in greater plasma leakage (McDonald et al, 1999).

If protein movement from the circulation was not restricted by its size the quantity leaking to the gut might be more dependent on the concentration gradient between blood and the gut. This may need to be considered when comparing excretion of proteins to the gut in patients with intestinal inflammation.

1.7.4 The source of IgG lost to the gut

IgG is the most abundant serum immunoglobulin class at 14 mg/ml. Although very little IgG is found in the intestinal lumen of healthy subjects, patients with intestinal inflammation show increased loss of IgG to the gut (Choudari et al, 1991) . These patients also lose albumin to the gut indicating that at some of the IgG is likely to come from plasma leakage. The relative coefficient of excretion of IgG compared to albumin may give an indication to the extent that plasma leakage contributes to WGLF IgG.

1.7.4.1 Evidence for local production of IgG in intestinal inflammation

The intestinal mucosa of patients with active IBD contains increased numbers of IgG B-cells in the lamina propria (Baklien and Brandtzaeg, 1975) whereas in inactive Crohn's disease the number of IgG B-cells is normal (Rosekrans et al, 1980). These IgG B-cells have been shown to produce IgG in culture of intestinal biopsies (Danis et al, 1984) or isolated lamina propria mononuclear cells (Wu et al, 1989), (MacDermott et al,1981) from patients with IBD. The increase in intestinal IgG might therefore be due to both an increased number of IgG producing cells and upregulation of IgG production by these cells. Intestinal IgG may play a role in exacerbation of intestinal inflammation.

1.7.4.2 Why are numbers of intestinal IgG B-cells increased in inflammation?

Differentiation of intestinal antibody producing cells under normal conditions

The pattern of cell adhesion molecules on circulating antibody producing cells has been shown to be dependent on the site of immunization. Healthy individuals were vaccinated with Salmonella Typhi and cell adhesion molecule expression on circulating lymphocytes studied by flow cytometry. Oral immunization resulted in nearly all the S.Typhi specific circulating antibody producing cells expressing $\alpha4\beta7$ with 45% of cells being positive for L-selectin. Perental immunization resulted in only 60% of antigen specific antibody forming cells expressing $\alpha4\beta7$ whereas 90% expressed L-selectin (Kantele et al, 1997). This suggests that the site of antigen engagement for naive lymphocytes determines their expression of cell adhesion molecules.

Immunohistochemical analysis for cell adhesion molecule expression on lymphocytes in the Peyer's patches, the site of lymphocyte priming in the gut has substantiated this. Naive T-cells in the high endothelial venules and naive B-cells in the follicle mantle both had high expression of L-selectin but only low expression of $\alpha4\beta7$. Contrary to this, memory T and B-cells in the afferent lymphatic area expressed more $\alpha4\beta7$ but with less L-selectin. This indicates that L-selectin is required for recruitment of naive cells into the Peyer's patches but upon lymphocyte activation, different cell adhesion molecules are upregulated (Farstad et al 1997).

B-cell blasts or plasma cells in the lamina propria express $\alpha4\beta7$, providing evidence that B-cells primed in the intestinal lamina propria are able to enter the site of antibody production in the intestine (Farstad et al, 1995). IgA producing cells are predominant in the intestinal lamina propria. Priming of naive B-cells in the Peyer's patches therefore directs antibody production towards IgA (rather than predominant IgG seen with priming in the peripheral lymph nodes) and upregulates adhesion molecules which appear to facilitate homing of these IgA producing cells back to the intestine.

L-selectin is expressed in high levels on naive lymphocytes and is involved in the initial tethering of these cells to vascular endothelium. In peripheral lymph nodes this involves association with carbohydrate residues on an addressin (adhesion molecule) specific to peripheral lymph nodes. In the Peyer's patch a different addressin, mucosal adhesion molecule-1 (MAdCAM-1), is expressed. This has similar carbohydrate units, allowing association with L-selectin bearing lymphocytes. Firm attachment of lymphocytes to the vascular endothelium of lymphoid organs appears to be similar for naive or memory cells, involving association of lymphocyte function associated antigen with the endothelial intercellular adhesion molecules (ICAM). However, in addition to adhesion, the extavasation of lymphocytes from the blood involves a selection process where lymphocytes may need to respond to chemical signals produced by the endothelium. Activated or memory lymphocytes might more readily respond to these signals.

The vascular endothelium of the intestinal lamina expresses a form of MAdCAM-1 that lacks the additional sugar residues expressed on MAdCAM-1 in the Peyer's patches. Naive lymphocytes do not usually enter the lamina propria whereas lymphocytes primed in the Peyer's patches that express $\alpha 4\beta 7$ do. Lymphocytes activated at other sites do not usually show high expression of $\alpha 4\beta 7$ and so do not enter the lamina propria.

Changes to lymphocyte homing that might occur in intestinal inflammation

The lamina propria of patients with IBD contains greatly increased numbers of IgG B-cells. There are two probable mechanisms for this, firstly, priming of naive B-cells in the Peyer's patches may be altered so that the emerging B-cell blasts express immunoglobulin γ-chain instead of α chain. This might involve an altered cytokine environment in the Peyer's patches. As B-cell blasts produced in the Peyer's patches are known to express $\alpha 4\beta 7$, the finding that a reduced proportion of lamina propria B-cells express this molecule does not fit with this hypothesis. (Yacyshyn et al, 1994).

The second hypothesis to explain increased IgG B-cells in the intestinal mucosa in inflammatory states is that the requirement of B-cells to express $\alpha 4\beta 7$ to enter the lamina propria is abrogated. This might be due to increased expression of other adhesion molecules such as ICAM-1 on the vascular endothelium (Bernstein et al, 1998) or expression of adhesion molecules that are normally specific for peripheral lymph nodes.

Immunohistochemical staining shows that most of the IgG B-cells in inflamed intestine are located deep in the mucosa, near to the blood vessels. This might suggest that IgG producing cells have been recruited from the blood (Baklien and Brandtzaeg, 1975). B-cells have which have been primed in peripheral lymph nodes preferentially produce IgG on re-encounter with their cognate antigen.

1.7.4.3 Specificity of intestinal IgG in patients with IBD

The antigen specificity of WGLF IgG might indicate whether the altered mucosal immune response in IBD is directed against a particular agent. Serological tests have demonstrated high frequencies of perinuclear antineutrophil cytoplasmic antibodies (pANCA) in UC. However, pANCA are found in patients with various autoimmune diseases, indicating that they may be secondary products of a chronic inflammatory response (Yang et al, 1993).

Many patients with Crohn's disease show raised serum IgG to bacterial antigens including anaerobic coccoid rods or a 45/48 Kd antigen cross-reactive with Mycobacterium (Oudkerk Pool et al, 1995). It is not known whether serum antibodies to intestinal bacteria are produced in the systemic compartment or reflect mucosal overproduction and leakage of these antibodies to the systemic compartment. IgG has no specific mechanism to transport it to the gut lumen and so if vascular permeability was increased, it may enter the circulation.

Under normal circumstances, the intestinal immune system does not produce IgG or cell mediated immune reactions to the commensal gut bacteria. However, any switch in the type of mucosal immune response might result in intestinal inflammation (because of positive feedback reactions resulting from trying to clear a large antigen load).

Evidence for abrogation of tolerance to gut bacteria in inflammation comes from a study looking at proliferation of isolated lamina propria mononuclear cells (LPMNC) in response to isolates of gut bacteria. Healthy individuals show no proliferation in response to their own gut bacteria but there is proliferation in response to gut bacteria from others. In contrast, LPMNC from patients with IBD proliferated in response to their own gut bacteria (Duchmann et al, 1995).

Evidence for an altered intestinal IgG immune response to commensal gut bacteria in patients with IBD comes from a study showing that IgG in endoscopic washings binds to commensal gut bacterial antigens isolated from faeces (Macpherson et al, 1996). By comparing IgG excretion to that of albumin, IgG was shown to be of intestinal origin. The specificity of IgG was examined by first separating the bacterial antigens by electrophoresis and then taking a western blot from this and probing using the endoscopic washing antibodies. IgG from serum or the intestine of control subjects was found to bind to both cytoplasmic and membrane bacterial antigens. In contrast, intestinal IgG from IBD patients bound preferentially to cytoplasmic bacterial antigens. This indicates that in the control group, IgG in the intestinal secretions and in serum are from the same source, possibly indicating a systemic immune response to bacteria that have crossed the intestinal mucosa. In IBD there appears to be an altered intestinal IgG response to commensal gut bacteria.

The IgG subclasses produced in the intestine may provide clues to the nature of the immune response. In normal subjects, a lower proportion of intestinal IgG cells are IgG1 (45%) compared to blood (70%). IgG2 is increased in the intestine and especially so in patients with Crohn's disease (Rüthlein, et al 1992). The proportion of intestinal IgG1 cells is increased in patients with ulcerative colitis (Iizuka, 1990). IgG2 is produced predominantly in response to particulate or carbohydrate antigens whereas IgG1 is produced in response to soluble or protein antigens.

1.7.4.4 How might IgG result in inflammatory damage to the intestine ?

Interaction with Fc receptors on immune cells

Fc receptors are cell surface receptors specific for the Fc portion of a particular class of immunoglobin. IgG Fc receptors are expressed constituatively on monocytes, macrophages, neutrophils, T cells and B cells. Type one receptors have greatest affinity for IgG1, IgG3 and IgG4 and are found on mononuclear cells. Type two receptors are of low affinity, bind IgG1 and 2, and are expressed on monocytes, neutrophils, eosinophils and B cells. Type 3 receptors are of intermediate affinity, bind IgG1 and IgG3 and are expressed on some monocytes and T cells or neutrophils and eosinophils. Tissue macrophages express all three types.

These receptors facilitate uptake of antigen in IgG immune complexes. This may induce phagocytic cells to release enzymes and oxygen radicals to destroy ingested antigen, possibly causing bystander damage. Antigen taken up via Fc receptors is efficiently presented to T-cells, augmenting the immune response (Van de Winkel and Anderson, 1991).

Interaction of IgG with complement

IgG immune complexes (containing IgG1 or IgG3) are efficient activators of the classical complement cascade. Under normal circumstances, IgA is the predominant immunoglobulin produced in the intestine. As IgA is not an efficient activator of complement this may prevent unnecessary inflammatory reactions.

Activation of the complement cascade within the intestinal mucosa may result in recruitment of neutrophils (via generation of anaphylotoxins C3a and C5a) and activation of phagocytes via C3b receptors. These cells produce reactive oxygen species and proteolytic enzymes in response to uptake of complement coated antigen. These can cause bystander damage to the surrounding tissue. Damage to the intestinal epithelium might allow increased amounts of antigen into the intestinal mucosa and this is likely to result in immune activation of local T and B lymphocytes. In individuals primed to a heightened immune reactivity, this could result in an amplified inflammatory relapse.

1.8 Complement in the gut

1.8.1 Overview of the complement system

Complement refers to a group of nine serum proteins (C1-C9) which can act as either an innate (the alternative pathway) or adaptive (classical pathway) anti microbial mechanism. The two pathways differ only in their dependence on the requirement for antibody in the initial activation step. The stages which follow form part of an amplification cascade in which proteins produced from one reaction act as catalysts for the next. The final result is polymerization of complement proteins within the target cell membrane, forming a pore which results in cell death by fluid uptake (Reviewed by Kinoshita, 1991)

1.8.1.1 The classical pathway

The first stage of complement activation requires antibody to be bound in an immune complex. Activation requires simultaneous binding of two globular heads of C1q to heavy chain domains of antigen complexed IgG or IgM. This requires two adjacent IgG or one molecule of IgM. C1q is then able to bind other components C1r and C1s, creating a serine protease. This enzyme acts on C4 to release a small peptide C4a and remain bound to the larger C4b fragment. C4b is a binding site for C2, which on binding is cleaved by the C1 protease to release C2b. C4bC2a is the classical C3 convertase.

As IgM can bind two molecules of C1q simultaneously, it would be expected that IgM is a more efficient activator of complement than is IgG. However, complement activation with IgM is less efficient than expected for two reasons: Firstly, C4b can not bind to IgM and so must be deposited on an acceptor (usually non host) membrane before further activation can occur. The IgM immune complex associated C1 must be close to the site of C4b deposition to increase the likelihood of C2a binding to it.

Secondly, antigen in soluble IgM immune complexes (antigen excess) is less efficient at activating complement than are IgM immune complexes in antibody excess (Van Der Zee et al, 1986). Antigen is usually in excess during the early stages of an immune response.

The classical C5 convertase

C3 convertase (C4bC2a) cleaves C3 to C3a and C3b. C3b has an exposed thio-ether bond which is very reactive and rapidly binds to proteins. C3b is deposited on nearby non-host cell membranes or upon the C4bC2a complex to form the C5 convertase.

1.8.1.2 Activation of the complement cascade by the alternative pathway

C3 slowly hydrolyses in solution, producing an activated molecule C3i. This is similar to C3b in that it binds to nearby cell membranes (particularly of bacteria) although stabilization of this requires binding of factor B. Bound factor B is cleaved by a serum enzyme, factor D, to release a small peptide, Ba. Membrane bound C3iBb is the alternative pathway C3 convertase which dissociates rapidly without binding of factor P. C3 convertase cleaves C3 to produce C3b which either binds to the non-host membrane (producing a focus for C3 convertase production) or another C3b to form the alternative pathway C5 convertase (C3bBbC3b).

1.8.1.3 Formation of the membrane attack complex

This sequence is common to both pathways and uses membrane deposited C3b as a focus for binding of proteins which are inserted into the cell membrane, resulting in cell lysis. This sequence is started by binding of C5 to the C3b of immobilized C5 convertase. Cleavage of bound C5 releases C5a, allowing binding of C6 to C5b and then C7 to C6. C7 has a hydrophobic tail which is inserted into the cell membrane. C8 then binds to C7, also embedding into the cell membrane. C8 allows insertion and polymerization of C9 within the cell membrane, resulting in a hole in the cell membrane with subsequent cell lysis.

1.8.1.4 Other biological functions of complement

Fragments C3a and C5a are chemotactic for neutrophils and trigger de-granulation of basophils and mast cells, with subsequent release of vasodilator substances which increase blood flow to the area. Several cells have receptors for C3b such as complement receptor 1 on monocytes or CR3 which binds the breakdown product of C3b, iC3b on monocytes and neutrophils. Uptake of antigen-complement complexes results in cellular activation, releasing enzymes and oxygen metabolites which help to destroy invading bacteria but can damage neighbouring tissues (Dahlgren et al, 1984).

1.8.1.5 Regulation of the complement system

Activation of complement is supported by immune complexes or non-host cell membranes. Activated complement components in the fluid phase are usually degraded by inhibitors e.g. C1 inhibitor. Host cell membranes do not support complement activation because an inhibitor, factor H, is able to facilitate cleavage of any bound C3b. On non host cells bound C3b binds factor B better than factor H.

Host cell membranes contain proteins which inhibit complement activation; decay accelerating factor (DAF), complement receptor 1 (CR1) and membrane cofactor protein (MCP). These inhibit the binding of C2 to C4b, promote the dissociation of C2a from C4b and promote the catabolism of C4b. DAF and CR1 also accelerates dissociation of the C3bBb C3 convertase. Another membrane protein, CD59, inhibits insertion and polymerization of C9 in cell membranes which have bound C5b-8 (Reviewed by Lachmann, 1991).

1.8.1.6 Evidence for complement activation in the inflamed intestine

Deposition of activated complement, (iC3b, the breakdown product of C3b), has been demonstrated by immunohistochemistry to occur along the intestinal epithelial basement membrane of biopsy specimens from patients with UC. Complement deposition was also shown in other types of colitis, indicating that this is not specific for UC (Ueki et al, 1996). In Crohn's disease, deposition of C3b at the luminal face of the surface epithelium was observed (Halstensen et al, 1992).

Deposited C3b was not associated with IgG indicating that intestinal complement activation was likely to be by the alternative pathway (Halstensen et al, 1992). Complement activation was not evident in the intestinal mucosa of healthy subjects.

1.8.1.7 Biosynthesis of complement components

The liver is the major source of complement proteins with some produced constituatively e.g. C7 whereas synthesis of others is upregulated during an acute phase response e.g. C3 (Morgan and Gasque, 1997). Patients who received a bone marrow transplant with a complement allele mismatch subsequently had a small proportion of serum C3 which had the marrow allele. Following liver transplatation, most of the recipients serum C3 bore the liver allele. This demonstrated that although the liver is the major source of C3, there is significant synthesis elsewhere (Naughton et al, 1996).

Several human cell types have, in culture, been shown to synthesize functional complement components. Monocytes produce C2, factor B and factor D (Beatty et al, 1981) and have been shown to synthesize C1-C5 in serum free culture (Hetland et al, 1999). Synthesis of C3 by alveolar macrophages was upregulated by endotoxin. Synthesis of C3 by intestinal macrophages might be expected to be upregulated in response to high levels of endotoxin in the intestinal lumen.

In the Caco-2 colonic epithelial cell line, production of C3, C4 and complement factor B was constitutive. Production of C3 was specifically increased in a dose dependent manner in response to by IL-1β or TNF-α (Andoh et al, 1993). As these cytokines were produced in the inflamed intestine, they might have increased epithelial complement synthesis. In the context of a local inflammatory reaction, the relatively small amounts of complement produced by these cells may have been significant. .

In vivo synthesis of complement has been demonstrated using a jejunal perfusion system. C3, C4 and factor B were quantified in the jejunal perfusate by ELISA. Patients with CD had greater complement secretion than healthy subjects, irrespective of their disease activity (Ahrenstedt. et al, 1990).

Comparison of complement excretion relative to albumin indicated that complement lost to the gut was likely to result from mucosal biosynthesis.

The drawbacks with this study are that only non-involved regions of the jejunum were studied. Complement secretion may have been greater in inflamed tissue. Measurement of complement in WGLF, a whole gut perfusion, may have given a more complete picture as well as allowing study of complement loss to the gut in ulcerative colitis, a colonic disease.

1.8.2 Methods for measuring complement in intestinal secretions

1.8.2.1 Measurement of complement functional capacity

The functional capacity of complement to undergo the classical amplification cascade is usually measured by the capacity to lyse antibody sensitized sheep red blood cells (Clark et al, 1985). The capacity of either the entire lytic sequence or a particular component can be measured, if purified components are supplied. These assays were designed for use in plasma and may not be suitable for WGLF; complement being dilute and possibly cleaved by gut proteases. Detection of individual complement component activity without the need for the intact cascade may have been useful; an ELISA format using immobilized IgG as a platform for complement activation with subsequent detection of fixed complement components has been described (Zwirner et al, 1989).

1.8.2.2 Immunological detection of complement components

C3 is the most abundant complement component in serum and is pivotal to the activation of both the classical and alternative pathways. C3 was therefore the most useful component to measure in a study of complement loss to the gut. How to measure C3 was dependent on whether total C3 or the extent of C3 conversion was required.

Detection of C3 conversion products

The most suitable fragment to examine was C3dg, the breakdown product of C3b. This was because antisera to C3b usually cross-react with C3. Problems with polyclonal antisera for C3dg which cross reacted with C3 were circumnavigated by electrophoretic separation of the complement fragments (Petersen et al, 1988). This required mg/ml concentrations of complement and so would be unlikely to work with WGLF. New assays for C3dg capture the molecule with monoclonal antibodies. This ensured that cross-reaction with C3 did not occur. (Peakman et al, 1987).
The extent of complement activation has been assessed from the ratio of intact C3 to C3d (Riordan et al, 1997). However, proteolysis of complement in WGLF may result in loss of the epitope that is detected by monoclonal antibodies to C3d.

Assay of total C3

Polyclonal antibodies against C3 also recognized C3b and C3c activation fragments (Porcel et al, 1993). These might therefore be useful for detecting different forms of C3 that might be present in WGLF. A capture ELISA with immobilized anti C3c as used with cell culture supernatants (Mitchell et al, 1996) and a competition ELISA using polyclonal anti C3c has been used with intestinal perfusion fluid and serum (Ahrenstedt et al, 1990).

The quantity of C3 detected is likely to represent a balance catabolism and synthesis (Porcel et al, 1993). In inflammation, synthesis of C3 is increased (Naughton et al, 1996) but complement activation and consumption may have been increased. C3 in intestinal secretions may reflect leakage of plasma proteins rather than mucosal synthesis. It may be useful to quantify C3 in serum in addition to WGLF to clarify whether C3 in the gut reflects plasma leakage or mucosal synthesis.

SECTION 2 MATERIALS AND METHODS

CHAPTER TWO

Methods to study the structural integrity of immunoglobulins in WGLF

2.1 Background

2.1.1 Investigating the structural integrity of immunoglobulins in WGLF

The validity of sandwich ELISA to quantify WGLF immunoglobulins depends on the assumption that WGLF immunoglobulins are intact. Antibodies to immunoglobulin heavy chain might detect both fragmented and intact immunoglobulins. Immunoglobulin fragments might show disproportionately greater binding of anti heavy chain as epitopes which are sterically inaccessible on the intact molecule may be revealed in the fragment. This could result in overestimation of total immunoglobulins, reducing the value of any conclusions. Conversely, ELISA to measure antibodies are dependent on an intact antigen binding site being associated with heavy chain. Fragmentation of these antibodies would result in loss of detection.

2.1.2 Previous work on WGLF immunoglobulin structure

Colleagues at the Gastrointestinal Laboratory, Edinburgh, have used SDS-PAGE followed by Western blotting to investigate the structural nature of immunoglobulins in WGLF. The finding that anti heavy chain staining of the western blot did not localize to a discrete band may have indicated that the immunoglobulins are fragmented. This result could have arisen because of cross-reactivity of the antisera with the myriad of proteins in WGLF. A preparative extraction stage prior to electrophoresis was required.

2.1.3 Methods for purifying immunoglobulins

Affinity chromatography using immobilized anti immunoglobulin heavy chain antibodies has often been used to purify immunoglobulins of a particular class (Mannik and Stage, 1971). Several methods are available for linking antibodies to cellulose chromatography resins e.g. Sepharose. However, the cyanogen bromide method (Axén et al, 1967) has been the most frequently used. Both intact and fragmented immunoglobulins bound to anti heavy chain. Affinity chromatography was therefore not suitable for separating intact from fragmented immunoglobulins.

2.1.4 Thiophilic ligand chromatography: a method which binds only intact immunoglobulins

A chromatography method which binds intact immunoglobulins of all classes, releasing them under gentle conditions has been described: "thiophilic absorption column chromatography"(Porath et al, 1985). Sepharose 4B gel which has been cross linked with divynyl sulphone and, blocked with 2-mercaptoethanol, possesses a thio-ether ligand (the resin was called T-Gel). In the presence of ammonium sulphate, immunoglobulins showed a high affinity binding to this ligand. Removal of the ammonium sulphate resulted in desorption of bound immunoglobulins from the column.

The authors claim that the recovery of immunoglobulins from colostrum was almost quantitative (Hutchens and Porath, 1986). It was demonstrated by size exclusion chromatography and SDS-PAGE that only intact molecules were binding (Hutchens et al, 1989). It may therefore be possible to separate intact from fragmented immunoglobulins using this technique.

The reactive ligand was of the form R-S-CH_2-CH_2-SO_2-CH_2-CH_2-O-polymer where R is a small aliphatic residue. The thioether sulphur and the sulphone groups needed to be in close proximity to maximize the thiophilic absorption of immunoglobulins. It has been suggested that an electron donar/acceptor mechanism or proton transfer between surface-accessible aromatic amino acids and the sulphone-thioether sulphur atoms in the T-Gel ligand might be responsible for the T-Gel interaction (Hutchens and Porath, 1997).

Immunoglobulin binding to the T-Gel was found to be enhanced by salts which are known to cause re-organisation of water molecules e.g., K_2SO_4. The interaction was shown not to be the same as hydrophobic chromatography as a hydrophobic column did not remove the same proteins as the T-Gel clolumn (Porath et al, 1985). It is thought that the protein T-Gel ligand association is stabilized by the displacement of protein bound water (Hutchens and Porath, 1997).

2.2 Work with T-Gel

2.2.1 Production of T-Gel

Sepharose 6B was 'suction-dried' using a sintered glass funnel attached to a vacuum pump. Dried Sepharose (100 g) was then suspended in 100 ml of 0.5 M sodium carbonate. To this, 5 ml of divynyl sulphone (Fluka) was added whilst stirring the suspension (this must be done in a fumehood). The Sepharose was then transferred to sealed universal tubes and mixed end over end, overnight at room temperature. This conjugated the sulphone group to the sepharose.

Unbound reagent was washed free by pouring the slurry into a sintered glass funnel and then flushing 2 litres of distilled water through it under vacuum. Divynyl sulphone treated Sepharose (80 g) was resuspended in 80 ml of 0.1 M sodium hydrogen carbonate pH 9. To this, 8 ml of 2- mercaptoethanol was added with mixing. The slurry was then mixed end over end in sealed universal tubes as before. This added the thioether sulphur and hydrocarbon residues to the end of the sulphone conjugated Sepharose.

Unreacted mercaptoethanol was washed free by pouring the slurry into a sintered glass funnel and then flushing with two litres of distilled water passed under vacuum. Prepared T-Gel was stored suction dried at 4°C in a sealed container containing silica gel desiccant.

In the original work the density of ligand in the final gel was assayed by analytic chemistry techniques (Hutchens et al, 1989). For this study the facility to do this was unavailable. The effectiveness of T-Gel ligand formation has been determined indirectly by assessment of immunoglobulin binding.

2.2.2 Testing the binding capacity of T-Gel for WGLF immunoglobulins

2.2.2.1 Reagents for T-Gel chromatography

a) T-Gel equilibrating buffer (promotes immunoglobulin binding).

20 mM HEPES buffer pH 8 containing 10% w/v ammonium sulphate, 0.5 M NaCl and 0.02% w/v sodium azide as a preservative.

b) T-Gel elution buffer (to release bound immunoglobulins).

Equilibrating buffer without the ammonium sulphate.

c) Ethylene glycol elute (facilitates complete elution)

50% ethylene glycol in 20 mM HEPES pH 8

d) T-Gel which has been pre-washed with 100 x volume equilibrating buffer before suction-drying.

All buffers were degassed in a side arm flask for 20 mins under vacuum (0.5 atmospheres).

2.2.2.2 Preparing WGLF for binding to T-Gel

WGLF (filtered and processed as described in the thesis appendix) was prepared for T-Gel as follows: Ammonium sulphate was added to a final concentration of 10% w/v, with sodium chloride added to a final concentration of 0.5 M. The pH was adjusted to 8.0 with 0.5 M NaOH.

2.2.2.3 Immunoglobulin binding to T-Gel

The binding capacity of the T-Gel for WGLF immunoglobulins was determined by measuring how much bound when varying quantities were added to a constant amount of T-Gel. Suction dried T-Gel (0.37 g) was dispensed into several screw top glass tubes. To one tube each, 2, 4, 8 or 16 ml of WGLF was added and the contents mixed by rotation for 30 mins at room temperature. Unbound material was separated from the gel by centrifugation at 1,850 x g for 10 mins, an aliquot of each supernatant being taken for total immunoglobulin analysis by class specific sandwich ELISA.

2.2.2.4 Removing unbound protein

The T-Gel was washed twice (resuspending in 5 ml of equilibrating buffer and then centrifugation at 1,800 x g for 10 mins). Removal of unbound protein was complete when the OD 280 nm of the supernatant equaled that for fresh equilibrating buffer.

2.2.2.5 Elution of bound protein

Bound protein was eluted by resuspending the gel in 5 ml of T-Gel eluting buffer and mixing by gentle inversion for 20 mins. Eluted material was separated from the gel by centrifugation at 1,800 x g for 10 mins. Supernatants were assayed for immunoglobulin. From the result of this experiment, the quantity of WGLF immunoglobulins to maximize T-Gel column binding was predicted.

2.2.3 Chromatography using T-Gel

2.2.3.1 Preparing a column

A Pharmacia 20 cm by 16 mm glass column was assembled as per manufacturers instructions. A polyamide/polypropylene bed support was fitted at the base and polyethylene tubing of one mm diameter attached to the column outlet. Air bubbles were flushed free from the bed support with equilibrating buffer expelled through a syringe. The outlet tube was then closed with a pin, retaining 2 ml of buffer above the net ring before clamping the column vertically in a retort stand.

2.2.3.2 Packing the gel

22 g of suction dried T-Gel was resuspended in 30 ml of equilibrating buffer. The slurry was poured in one motion along the edge of a glass rod and into the column. This allowed the gel to settle evenly without introducing air. An inlet column adapter type C 16 (Pharmacia) was assembled as per manufacturers instructions and then inserted into the top of the column. Air was displaced from the inlet tubing by pushing the adapter down into the buffer head above the gel. The adapter was then sealed in position, closing the 'O' ring by means of an adapter screw.

The column inlet adapter was then connected using 1 mm polyethylene tubing to the peristaltic pump (Pharmacia P1, Sweden, with 2.1 mm diameter rubber pump tubing). Pump inflow tubing was secured in a reservoir of equilibrating buffer and the column outflow sealed with a pin to prevent the column from drying out.

To pack the gel, the column outflow was released and T-Gel equilibrating buffer was pumped onto the top of the column at 130 ml/h for 45 mins. Once the bed height had dropped to a constant level, packing was completed. The column adapter was repositioned on the gel surface to ensure even sample loading. The flow rate of the pump was checked by measuring the volume of buffer passed through the column (collected in a measuring cylinder) during a set time period. Forward flow (from top to bottom of the column) was used unless otherwise stated.

2.2.3.3 Samples for chromatography

WGLF was selected from patients with IBD as these usually had high immunoglobulin content. WGLF from a different patient was used for each experiment. For studying serum immunoglobulins, a pool of serum from several patients was used. Serum was filtered through a 0.22 μl cellulose filter (Millipore) before use in chromatography. All samples were treated as follows: Ammonium sulphate was added to 10%, NaCl to 0.5 M and the pH adjusted to 8.0 with NaOH.

2.2.3.4 General conditions for chromatography

For preliminary experiments (1-3) chromatography was performed at room temperature with the loading sample being kept on ice and collected fractions being transferred to storage at 4°C. During these experiments the influence of the rate of loading and the sample volume were assessed. Later a cold room at 4°C was used. WGLF was stored at -70°C and thawed once prior to chromatography(unless stated).

In the original T-Gel work, immunoglobulin binding was unaltered on changing the temeperature from 4°C to 20°C (Hutchens and Porath, 1986). Immunoglobulin binding at 4°C could be improved by reducing the flow rate. This was considered before selecting optimal chromatography conditions.

2.2.3.5 Chromatography stages

Sample was loaded onto the top of the column, with the column outflow released to allow the sample to progress though the gel. The progress of WGLF through the gel was followed by the presence of a yellow band (probably bilirubin). This gave a sharp front at an even height around the column, indicating that the gel had no 'channels' and so had been properly packed. The void volume of the gel was the volume of WGLF loaded when the yellow band became visible in the column outflow.

After sample loading, loosely bound proteins were removed by flushing the column with T-Gel equilibrating buffer. The protein content of the column washings was monitored spectrophotometrically at 280 nm (the OD 280 nm is an indirect measure of protein concentration as aromatic amino acids absorb light strongly at this wavelength). Washing was stopped when the OD 280 nm of the column washings equaled that for fresh equilibrating buffer

Strongly bound proteins were released by pumping T-Gel elution buffer through the column. Fractions were collected into siliconised glass tubes, at fixed time intervals, using a fraction collector (Gilson Microcoll, France). Fractions were stored at 4°C. For some experiments, tightly bound immunoglobulins were released by the addition of ethylene glycol to the eluting buffer, either as a single step or after a preliminary elution with elution buffer alone. The profile of protein elution was monitored by measuring the OD 280 nm or quantified using the Biorad dye binding version of the Bradford assay (described in the thesis appendix).

Total immunoglobulin were assayed by ELISA as described in the thesis appendix. Individual fractions were first assayed at a single dilution. From these results fractions with similar protein content were pooled and then assayed at doubling dilutions for quantification.

The quantity of immunoglobulin was calculated from concentration x the fraction volume (this being estimated from the difference in weight between the fraction and the empty fraction tube). Buffer density at 20°C was:

Equilibrating buffer 1.07 g/ml

Elution buffer 1.03 g/ml

Elution buffer including 50% ethylene glycol 1.08 g/ml

2.2.3.6 Re-cycling of T-Gel

After each experiment, 60 ml of 0.1 M NaOH was flushed through the column at 48 ml/h to disrupt the binding of any residual protein. T-Gel equilibrating buffer (90 ml) was then pumped through the column at 48 ml/h. After two chromatography runs the gel was either resuspended and the column repacked or unused T-Gel used.

2.3 SDS-Polyacylamide gel electrophoresis

SDS-PAGE has been extensively for resolving protein mixtures according to molecular weight. This was used to analyse WGLF proteins fractionated by T-Gel chromatography.

2.3.1 Equipment, reagents and gel formation: see appendix

2.3.2 Sample preparation

T-Gel fractions of WGLF proteins from experiment 1 had high concentrations of IgG, IgM and IgA and were selected for structural analysis. Pools of those fractions with highest immunoglobulin content were made for the loading, washing and elution stages. Fractionated immunoglobulins (10 ml) were dialysed in a Spectra-Por (Medicell International, UK) 3,500 mw membrane sac, overnight at 4°C against two changes of 0.125 M Tris-HCl pH 6.8 (100 ml). Immunoglobulins were then concentrated overnight using osmotic pressure at 4°C (Millipore Urofil, USA). In this system, sample is loaded into a chamber which is isolated from chamber from a sponge by a 7 Kd mw cut off selectively permeable membrane.

Water is absorbed into the sponge, concentrating the protein. Unfractionated WGLF was concentrated five fold and fractionated immunoglobulins concentrated ten fold.

2.3.2.1 Reduction of disulphide bridges in the protein samples

Samples were mixed 1:2 with reducing gel buffer (6% SDS, 6% 2-mercaptoethanol, 40% sucrose and 0.02% bromophenol blue in 0.125 M Tris-HCl pH 6.8), before boiling for four minutes.

2.3.3 Running the electrophoresis

Reduced samples (30 µl) or 5 µl of molecular weight protein standards (Sigma) were separated by electrophoresis overnight at room temperature, with constant current of 30 mA supplied by powerpack (Shandon, UK). Electrophoresis was stopped when the bromophenol blue dye had migrated to within 1 cm of the base of the gel.

2.3.3.1 Gel staining with Coomassie brilliant blue

The gel had been loaded with the same samples in more than one location. One part of the resolving gel was taken for protein staining with Coomassie blue, as described in the thesis appendix. The other half of the gel was used for western blotting with subsequent antibody staining.

2.3.3.2 Observation of the staining pattern

For both the test samples and the molecular weight standards, the distance moved by the protein band from the top of the resolving gel was compared to the distance moved by the bromophenol blue dye front. This ratio, the Rf, was plotted against the $\log_{10}$ of the molecular weight for the standards. From this plot, the molecular weight of the sample protein bands was interpolated.

2.4 Western blotting

SDS-PAGE separated WGLF proteins were transferred to a nitrocellulose membrane by western blotting. Commercial antisera against human immunoglobulins were then used to identify these proteins.

2.4.1 Equipment, reagents and assemby of blotting apparatus

see appendix

2.4.2 Testing the immunoglobulin detection system

The suitability of antibodies to detect immunoglobulins was first tested on small pieces of membrane onto which immunoglobulins had been absorbed. Nitrocellulose (0.45 μm pore, Whatman, UK) was cut into 0.25 cm^2 squares which were placed in separate compartments of a tissue culture plate. Nitrocellulose was activated by wetting with distilled water, excess fluid then being aspirated. Purified human colostrum (1 μl) was applied to the corners of the square each at 2.3 mg/ml, 0.23 mg/ml and 0.023 mg/ml of IgA to test the detection antibody sensitivity. Unoccupied protein binding sites on nitrocellulose were blocked by incubating with a 3% w/v solution of fat free milk in TBS-Tween for 30 mins, room temperature (with gentle rocking).

2.4.2.1 Detection system for IgA

Two systems were tested

Direct system: Goat anti human alpha chain alkaline phosphatase conjugate (Sigma)

Two antibody system: Goat anti human alpha chain (KPL) then rabbit anti goat Fc alkaline phosphatase conjugate.

Reagents

a) Tris buffered saline (TBS):

10 mM Tris and 140 mM NaCl adjusted to pH 7.4 with 1 M HCl.

b) TBS-Tween:

TBS containing 0.1% (v/v) Tween 20.

c) Blocking solution:

3% low fat milk (Asda) in TBS-Tween.

Prepare fresh on the day, dissolving milk by stirring in distilled water first.

d) Antiserum

e.g. Sigma goat anti human IgA (α chain specific) alkaline phosphatase conjugate.

e) Substrate:

For alkaline phosphatase use Sigma Fast BCIP/NBT phosphatase substrate one tablet in 10 ml distilled water is enough to stain a 12 x 5 cm piece of nitrocellulose.

2.4.2.2 Detection systems for IgG and IgM

Using a dot blot system as described for IgA, SPS-01 containing IgG and IgM was spotted onto nitrocellulose and detected using different antibody concentrations or incubation times. Optimum conditions giving clear staining with low background were selected for use with western blots.

For IgG, Sigma anti human γ chain, alkaline phosphatase conjugate was used.

For IgM, Sigma anti human μ chain, alkaline phosphatase conjugate was used.

2.4.3 Running the transfer

A maximum recommended initial current of 2.5 mA/cm^2 (370 mA for a 15 cm x 10 cm gel sandwich) was used. A transfer time of one hour rather than the recommended thirty minutes was used because of the gel thickness (1.5 mm) and high percentage acrylamide (12.5% w/v).

2.4.3.1 Confirming that protein transfer had occurred

Following transfer, the nitrocellulose sheet was placed in a plastic box and rinsed by submersion in TBS-Tween for two minutes. After decanting the wash, protein was visualized by addition of Ponceau S solution (Sigma, UK) diluted to 1/10 in distilled water (which gives a pink stain with protein). Background staining of the nitrocellulose was reduced by washing the nitrocellulose in TBS-Tween.

2.4.4 Probing of western blot with anti human α,γ and μ chain antisera

The nitrocellulose blot was divided into three, marking the gel running direction on each with a small cut at the base (anode) end. Each section was placed in a plastic box and unoccupied protein binding sites on the nitrocellulose were blocked by bathing nitrocellulose in a solution of 3 % w/v fat free milk in TBS-Tween for one hour. Immunoglobulins were detected using a single antibody system with Sigma alkaline phosphatase conjugated goat anti human immunoglobulin heavy chain diluted in TBS-Tween (15 ml). This was incubated for 1.5 hours at rt with rocking.

IgG use anti γ chain at 1/1,500
IgA use anti α chain at 1/2,500
IgM use anti μ chain at 1/2,500

Detection of bound antibody

Unbound antibody was aspirated by pipette and the nitrocellulose washed with four times two minute washes with TBS-Tween. Sigma "Fast" BCIP/NBT substrate was prepared by dissolving one tablet in 10 ml of distilled water. Substrate was then poured onto the nitrocellulose. Clear purple staining of discrete bands with low background was obtained, at which point the reaction was stopped by washing away surplus substrate with TBS Tween. The blot was dried on filter paper and stored in a sealed polythene bag in the dark.

The enzyme reaction involved cleavage of the phosphate group from 5-Bromo-4-Chloro-3-Indolyl Phosphate to allow coupling to Nitro Blue Tetrazolium. This produced a purple precipitate.

CHAPTER THREE

Development of an assay to quantify complement C3 in WGLF

3.1 Introduction

WGLF IgG has been validated as a useful marker of disease activity in patients with active IBD (Choudari et al, 1993). Increased IgG in the intestine may in itself have exacerbated mucosal inflammation. One possible mechanism is via activation of the complement system (Lake et al, 1975). IgG in immune complexes is an efficient activator of the classical complement pathway. Deposition of activated complement may directly damage mucosa cells or, chemotactic metabolites C5a and C3a recruit activated neutrophils to the region.

If complement is to play an important role in the exacerbation of intestinal inflammation, the first phase of study into this should look to detect complement in the gut. As C3 is the complement component at highest concentration in serum and is one which is pivotal to amplification of both the classical and alternative pathways, C3 would be a logical component to study in WGLF in relation to intestinal inflammation.

3.1.1 The possible state of C3 in intestinal secretions

C3 may be converted to fragments by the specific activation pathways which may occur during transit across the gut wall or within the intestinal lumen. Non specific proteases of pancreatic or bacterial origin may also increase complement conversion or further digest complement fragments. In active IBD, increased numbers of granulocytes in the intestine might have release enzymes which subsequently digest proteins, including complement (Johnson et al, 1976). Proteolysis of complement might result in underestimation of its biological activity.

Conversely, immunological detection of complement may give an overestimation due to detection of fragments. Consideration must therefore be given to the choice of a suitable method for quantifying C3 in WGLF.

3.2 The possible methods of analysis for C3 in WGLF

3.2.1 C3 fixation

Assay of the full classical pathway by haemolysis of antibody sensitized sheep red blood cells may not be useful for two reasons:

1) WGLF may contain intact C3, but without the other complement components formation of the membrane attack complex will not occur.
2): Only one biological activity of complement is estimated i.e. that of full activation. C3 conversion contributes much to the biological activity of complement through the generation of the chemotactic fragment C3a which attracts neutrophils to the site of inflammation (Dahlgren et al, 1984). C3b coated immune complexes facilitate the uptake of antigens by phagocytes with possible presentation of this antigen to T and B-cells .

A method to measure intact C3 by measuring its capability for conversion might be useful. In one assay, complement activation was promoted on immobilised IgG and bound C3 is subsequently detected immunologically (Zwirner et al, 1989). Complement fixation was optimized by using heat aggregated IgG (Maillet et al, 1992). It was decided to try the method of Zwirner et al with the adaptation of using aggregated IgG. A further adaptation was to include magnesium and cofactors in the sample buffer. These were necessary for classical complement activation but may have been bound to EDTA, a chelating agent used in WGLF processing.

3.2.1.1 Assay to quantify unspent C3 in WGLF

Reagents (from Sigma unless stated)

Coating buffer: 0.05 M carbonate/bicarbonate pH 9.6

Aggregated human IgG: Purified IgG was diluted to 20 μg/ml in coating buffer. This was heated in a waterbath at 63°C for 30 mins and then insoluble complexes removed by centrifugation at 250 x g for 10 mins. The supernatant was taken for use as the coating antigen.

Wash buffer: 0.05% Tween 20 in 0.9% saline

Blocking buffer: 1% BSA in wash buffer

Sample buffer: 0.15 M Tris pH 7.5 containing 1.2 mM $CaCl_2.2H_2O$ and 0.6 mM $MgCl_2.6H_2O$.

Standard: Random plasma/EDTA was chosen as an arbitrary C3 standard.

Samples: Filtered and processed WGLF (as described in the appendix).

Anti C3: Goat IgG fraction, anti human C3.

Anti goat conjugate: Rabbit anti goat IgG, alkaline phosphatase conjugate.

Protocol (All volumes 100 μl unless stated)

See appendix section on equipment for ELISA

1. Dynatech Immulon 1 plates (USA) were coated with aggregated human IgG and then incubated overnight at 4°C.
2. Unbound material was removed by three wash cycles with wash buffer.
3. Unreacted binding sites on the plate were blocked by filling the wells with block solution and incubating for 1.5 h at 24°C
4. Standard was diluted to 1/100 in sample buffer before adding to the plate in doubling dilutions.
5. WGLF was diluted to 1/5 in sample buffer before adding to the plate in doubling dilutions.
6. After addition of standard and samples, the plate was incubated for 2 h at 37°C.
7. Unbound material was removed by three wash cycles with wash buffer.
8. Bound C3 was detected by addition of anti C3 diluted in wash buffer. The plate was incubated for 2 h at 37°C.

9. Unbound anti C3 was removed by three wash cycles with wash buffer.
10. Bound anti C3 was detected by addition of rabbit anti goat conjugate, diluted to 1/5,000 wash buffer. The plate was incubated for 2 h at 37°C.
11. Unbound rabbit anti goat was removed by three wash cycles with wash buffer.
12. Bound alkaline phosphatase conjugate was detected with the substrate p-nitrophenyl phosphate (see thesis appendix), reading the OD at 405 nm.
13. A standard curve of OD against dilution was drawn and the response curve for WGLF compared against this.

Other minor modifications to the original method have been to make use of readily available or more cost-effective materials. This has been validated by the successful detection of fixed C3 from EDTA-plasma (see results).

3.2.1.2 The effect of protease inhibitors on complement fixation

The possibility of inhibition of complement fixation by protease inhibitors used in the processing of WGLF was investigated. In a series of preliminary studies, plasma-EDTA was processed using various combinations of SBTI, PMSF and NBCS as used for WGLF.

3.2.2 Detection of total C3

Total C3 could be quantified using a polyclonal antisera against C3c, which recognizes C3, C3b and C3c. Potentially useful assays included the double antibody sandwich or competition ELISA. An advantage of a competition ELISA was that only one antibody binding site is necessary for detection of the antigen. This removed the possibility that the capture antibody engaged all the possible epitopes required for detection with the second antibody. A potential disadvantage of the competition ELISA was that fragments of C3 or C3c might have lead to overestimation of C3.

A competition ELISA had been used to quantify C3c in intestinal secretions (Ahrenstedt et al, 1990) but no validation data was included. It was therefore decided to develop and validate a similar competition ELISA for WGLF C3c.

3.2.2.1 Development of competition ELISA for C3

Immobilized purified C3 competes with sample C3 for binding of polyclonal rabbit anti human C3c. Anti C3c which is bound to the plate is detected with goat anti rabbit alkaline phosphatase conjugate.

3.2.2.2 Optimization of assay

a. Concentration of antibody conjugate required to measure anti C3c

Dynatech Immulon 2 plates were chosen for their high percentage binding of protein antigens. These were coated with 100 µl/well of anti C3c at different dilutions for each column: 1/500, 1/1,000, 1/2,000, 1/4,000, 1/7,000, 1/10,000, 1/15,000, 1/20,000, 1/30,000, 1/40,000 and 1/50,000 in carbonate/bicarbonate pH 9.6. Binding was completed on incubation for 3 h at 37°C. After washing 3x in saline/Tween, unreacted binding sites were blocked using 1% w/v BSA in saline/Tween for 1 h at 37°C.

Block solution was decanted and goat anti rabbit IgG alkaline phosphatase conjugate added (100 µl/well) at different dilutions for each row: 1/250, 1/500, 1/1,000, 1/2,000, 1/3,000, 1/5,000, 1/7,500 and 1/10,000. The plate was then incubated for 3 h at 24°C, washed, and the colour developed with p-nitrophenol phosphate substrate. The OD 405 nm was read when the maximum reading reached 1.000.

b. Concentration of C3 for coating plate

An Immulon 2 plate was coated with purified human C3 at concentrations from 0.05 µg/ml to 10 µg/ml. Bound C3 was detected with anti C3c at dilutions from 1/2,000 to 1/5,000 followed by anti rabbit conjugate at 1/500.

c. Further adjustment of conjugate antibody concentration.

An Immulon 2 plate was coated with human C3 at 3 μg/ml and then blocked as before. Anti C3c (100 μl) was added in doubling dilutions from 1/1,000 to columns across the plate. After incubation and washing, goat anti rabbit IgG, alkaline phosphatase conjugate (100 μl) was added in doubling dilutions from 1/1,000 down the plate.

d. Concentration of anti C3c to compete with added C3

For a competition ELISA, it is important that the detection antibody (in this case anti C3c) is not in excess. Using a constant amount of immobilized C3, competing C3 was diluted with varying quantities of anti C3c. Competition curves were then drawn and the anti C3c concentration that gave optimum sensitivity was selected.

To a plate coated with C3 at 3 μg/ml, C3 (50 μl) was added at doubling dilutions from 10 μg/ml. Competing C3 had been treated similarly to that coated on the plate, i.e., it had been incubated overnight at 4°C. Anti C3c (50 μl) was added to duplicate columns in doubling dilutions starting from 1/2,000. The plate was incubated at 37°C for two hours to facilitate antibody binding. After washing free unbound anti C3c, anti rabbit conjugate was added at 1/16,000 and the plate incubated for two hours at 37°C. After washing unbound conjugate free, bound antibody was detected with the p-nitrophenyl substrate reaction (thesis appendix).

e. The state of the competing antigen - influence of duration of thawing

In a parallel experiment, purified C3 that had been freshly thawed was used as the competing antigen and compared with antigen thawed overnight.

3.2.2.3 Competition with WGLF C3 and recovery of purified C3 from WGLF

To a C3 coated plate, filtered and processed WGLF (50 μl) was added at a dilution of 1/2 and then doubly diluted. In parallel, the same samples at 1/2 with the inclusion of purified C3 (2.5 μg/ml) were added to the plate and doubly diluted.

As a standard, purified C3 (50 µl) was added to the plate in doubling dilutions from 5 µg/ml. Anti C3c was diluted to 1/32,000 in two stages; 5 µl + 1595 µl saline, mix then take 100 µl and dilute to 10 ml with saline. Diluted anti C3c (50 µl) was added to all wells except the blanks (a true 'blank' in the competition assay was when no detection antibody binds to the immobilized antigen). As it is expensive to use an infinitely high concentration of competing antigen, the cheaper alternative was to omit detection antibody as the blank). After incubation, bound anti C3c was detected with anti rabbit IgG at 1/16,000.

3.2.2.4 Influence of gut proteases on detection of C3 in WGLF

The protease activity of unprocessed WGLF was measured using the Azocoll dye release method (see appendix). C3 was assayed in the corresponding filtered processed specimens. If proteolysis during gut transit influenced the detection of WGLF C3, it would be expected to see a relationship between protease activity of unprocessed WGLF and the quantity of C3 detected.

3.2.2.5 Quantifying C3 in serum

Method as for WGLF except that serum was doubly diluted from 1/500

3.2.2.6 Assay performance

a) WGLF C3 limit of detection (sensitivity)

b) Quality control

An aliquot of WGLF (stored at -20°C), was tested on each plate, using duplicate doubling dilutions from 1/2 to 1/16.

1) Within batch variation (WGLF)

A single WGLF sample was diluted 1/2 in 12 separate tubes and these treated as separate samples, being doubly diluted to 1/8 in a C3 competition ELISA plate, and the coefficient of variation (CV) determined.

2) Between batch variation (WGLF)

WGLF C3 QC material was measured in 26 separate analytical runs and the CV determined.

3) Within batch variation (serum)

A single serum sample was diluted into 12 separate tubes at 1/500. These were tested as single samples, diluting on the plate to 1/2,000.

4) Between batch variation (serum)

Thirteen serum samples were analysed for C3 on two separate occasions. The samples were frozen and thawed for a second time as additional aliquots were unavailable. The standard deviation for the duplicate results was obtained from: $SD = \sqrt{\sum d^2/n}$ where d = difference between duplicate values and n = number of pairs.

3.3 RESULTS

3.3.1 Modifications of fixation assay for WGLF

TABLE 3.1 Effect that addition of WGLF processing reagents to plasma had on the detection of C3 fixation

Sample	OD 405 nm for C3 fixation	% inhibition
Plasma alone	0.959	
Plasma + SBTI	1.061	0
Plasma + PMSF	0.463	52
Plasma + NBCS	0.033	97
Plasma + SBTI + PMSF	0.648	32
Plasma + SBTI + NBCS	0.067	93
Plasma + 1/100 PMSF	0.845	12
Plasma + 1/100 NBCS	0.819	15

Protease inhibitors soyabean trypsin inhibitor and phenyl methyl sulphanyl fluoride or newborn calf serum (a source of non human complement) were added separately and in combination to plasma, using the same quantities as for WGLF processing as described in the thesis appendix. The possible interference on C3 fixation was then assessed by assay of C3 fixation in both processed and unprocessed plasma.

These results show that reagents used for processing WGLF interfered with the ability of the fixation assay to detect unspent C3 in serum and this may have also been the case with WGLF. PMSF is a serine protease inhibitor which may have inhibited the activity of activated C1s, a serine protease. This would have reduced the conversion of C4 and subsequent formation of the classical pathway C3 convertase. Interestingly soyabean trypsin inhibitor, another serine protease inhibitor, had no effect, indicating that conversion of C3 in this assay is via a specific pathway and not via the action of other proteases. Newborn calf serum (NBCS) gave almost complete inhibition of C3 fixation, possibly due to competition of bovine C3 for binding sites resulting from classical pathway activation on the immobilised IgG complexes. The antisera detecting human C3 will not detect bound bovine C3. In addition, human serum might have contained antibodies to bovine proteins (e.g. food antigens). These might form immune complexes with subsequent fixation of C3 that was not detected in the assay.

3.3.1.1 Alteration to WGLF processing

To further test fixation of C3 from WGLF, three WGLF samples were processed without PMSF and NBCS. As an alternative protein substrate for residual proteases, bovine serum albumin (BSA) was added to 2 mg/ml.
No C3 fixation was observed so it was assumed that C3 in WGLF was spent.

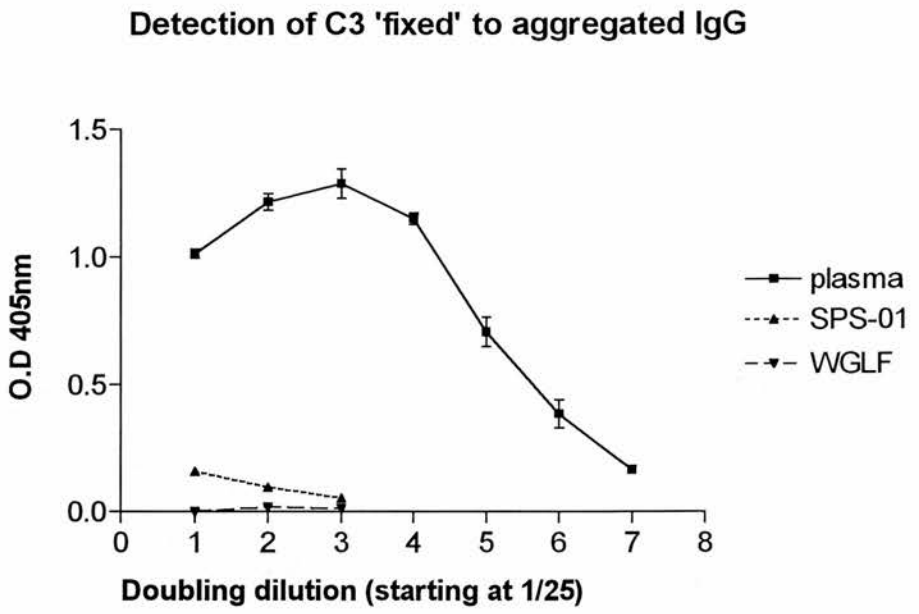

Fig 3.1: Classical pathway activity of plasma, serum and WGLF

Samples were added to 2 μg of aggregated human IgG and incubated for 2 h at 37°C to allow classical pathway activation to occur. Fixed C3 was detected using goat anti human C3 followed by alkaline phosphatase conjugated rabbit anti goat.

C3 fixation was seen with plasma but not with WGLF. As the extrinsic inhibitors of complement fixation had been removed by alteration of the WGLF processing regimen, it was likely that C3 fixation did not occur because complement in WGLF was spent.

3.3.2 Optimization of the competition ELISA for C3

3.3.2.1 Conjugate antibody

When the OD values were plotted against coating antibody dilutions, different response curves were seen with different amounts of conjugate antibody. At conjugate dilutions greater than 1/500 the slope of this response curve was reduced, indicating a loss of sensitivity. A conjugate strength of 1/500 seems optimal as high background colour is apparent at greater concentration (Fig 3.2).

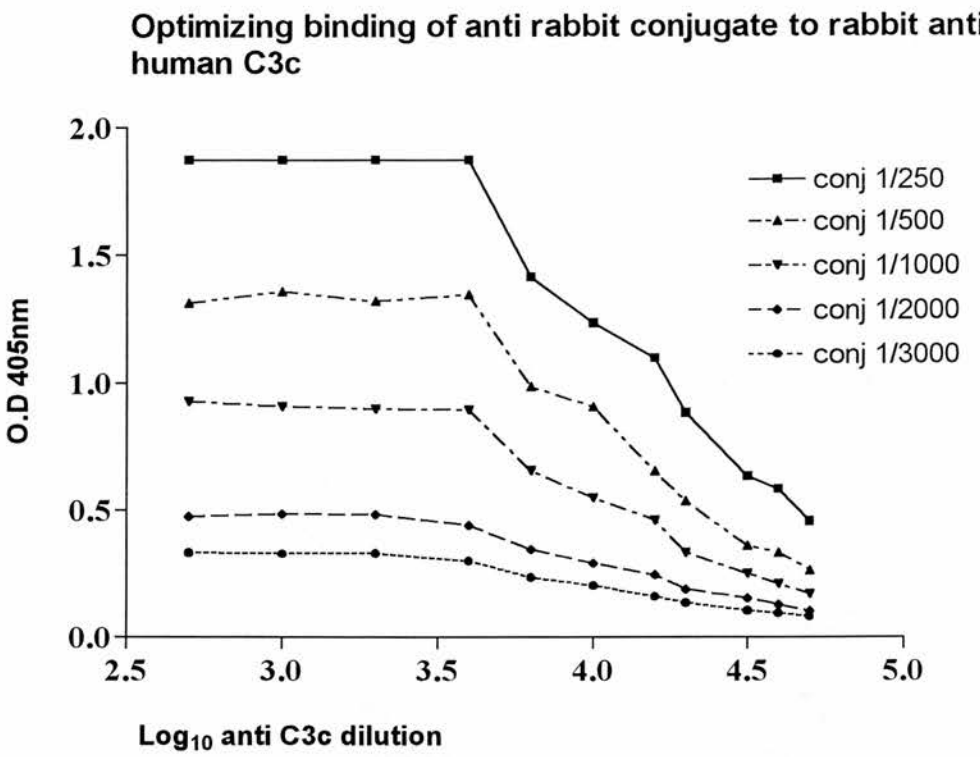

Fig 3.2: Optimization of the quantity of goat anti rabbit alkaline phosphatase conjugate required to detect rabbit anti human C3c absorbed onto plastic.

100 µl of varying concentrations of anti C3c were absorbed onto the wells of an Immulon 2 ELISA plate, unbound material being washed free. Absorbed anti C3c was detected using 100 µl of anti rabbit conjugate at different concentrations. A log plot of anti C3c concentration simplifies plotting a wide concentration range by condensing the graph. A $\log_{10}$ dilution of 3.0 is intended to represent a dilution of 1/1,000. The conjugate strength giving a wide range in signal with low background was chosen as optimal.

The plateau in the response curves occured with an anti C3c dilution of 1/2,000. This was thought to be a suitable concentration for detecting purified C3 in the competition assay.

3.3.2.2 Coating

A concentration of C3 of between 2.5 and 5 µg/ml gave the maximum signal (Fig 3.3), in agreement with 3 µg/ml used by Ahrenstedt et al, 1990.

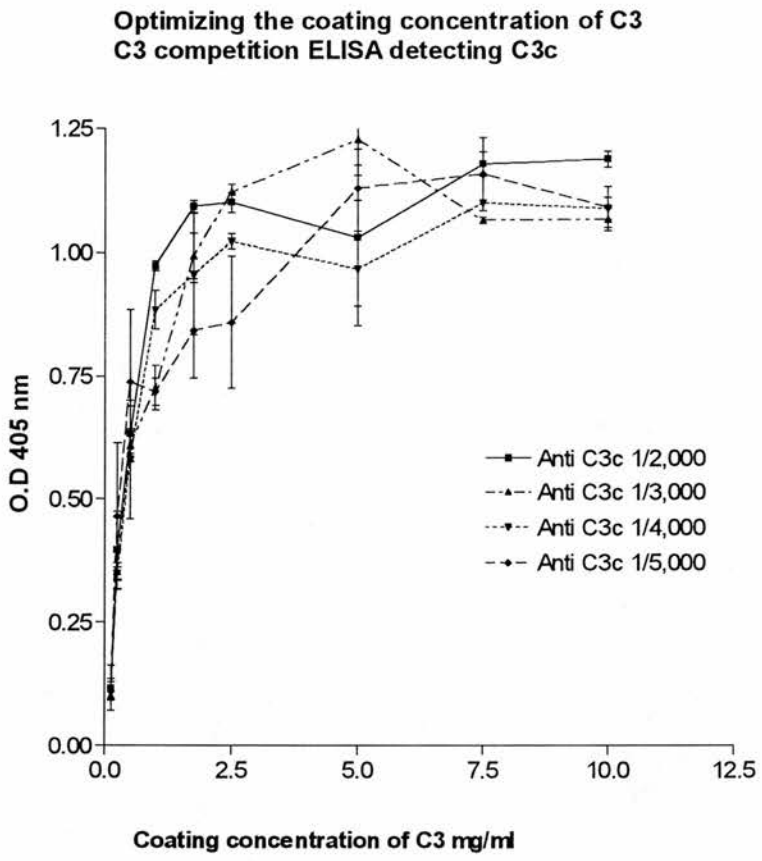

Fig 3.3: Optimizing the C3 coating antigen concentration for the competition ELISA to detect C3

100 µl of human C3 was absorbed onto the wells of an ELISA plate. Bound C3 was detected with 100 µl of rabbit anti human C3c at four different concentrations. Bound antibodies were then detected with alkaline phosphatase conjugated goat anti rabbit at 1/1,000.

The colour developed extremely quickly (1 min), compared to a required optimum of ten to fifteen minutes for a peak OD of 1.00. Fast development could lead to differences in the OD readings across the plate being due to the time taken to add the substrate from one side to the other. When anti C3c was previously absorbed directly onto plastic at 1/5,000 (fig 3.2), binding of alkaline phosphatase conjugated rabbit anti goat was sub-optimal giving an OD reading 80 % of the maximal. However, when anti C3c was used to detect immobilised antigen, there was no evidence to suggest that a dilution of 1/5,000 is sub-optimal.

This indicated that the primary antibody, anti C3c was more efficiently detected by the anti goat conjugate when the primary antibody is bound to an immobilized antigen i.e. as it was in the competitive ELISA. The optimal conjugate dilution should therefore be assessed under those conditions.

3.3.2.3 Further optimization of the conjugate strength

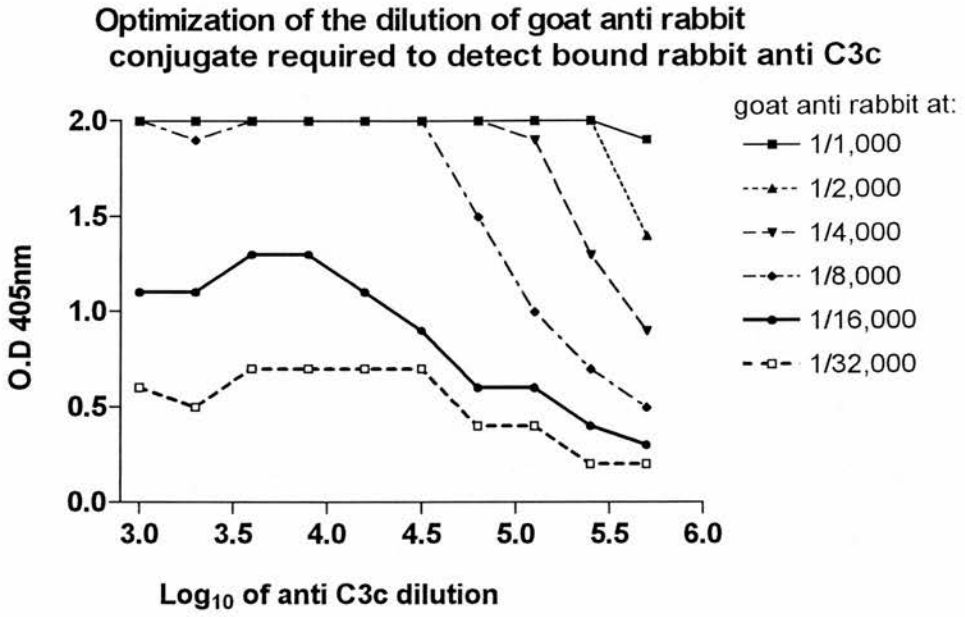

Fig 3.4: Further optimization of the conjugate strength in the C3 competition ELISA

An Immulon 2 ELISA plate was coated with 100 µl/well human C3. This was detected with 100 µl of rabbit anti human C3c used at a wide range of dilutions from 1/1,000 to 1/1,024,000. Bound anti C3c was then detected with alkaline phosphate conjugated goat anti rabbit.

The criteria selected for choosing the optimum conjugate antibody strength are that the colour reaction occurs within a reasonable time (OD of 1.0 in 10-30 minutes), that the conjugate differentiates a wide concentration range of bound primary antibody and that the background colour is low.

Only a conjugate concentration of 1/16,000 fulfilled all three criteria. This concentration makes the assay less sensitive to small changes in anti C3c binding but, possibly more robust.

3.3.2.4 Optimizing concentration of anti C3c for competition against added C3

a) effect of anti C3c concentration

Result

A competition type dose response curve was seen with anti C3c at 1/32,000 and to a lesser degree at 1/16,000. The former gave a maximum optical density of 1.0 in 19 mins. Anti C3c at 1/32,000 was chosen as optimal to detect competition between immobilised and competing C3. This fits in with the finding in Fig 3.4 that anti C3c at concentration greater than 1/10,000 gives a response in the plateau area of the curve i.e., anti C3c is in excess. At this antibody concentration, addition of competing C3 was unlikely to modulate binding of anti C3c to immobilised antigen.

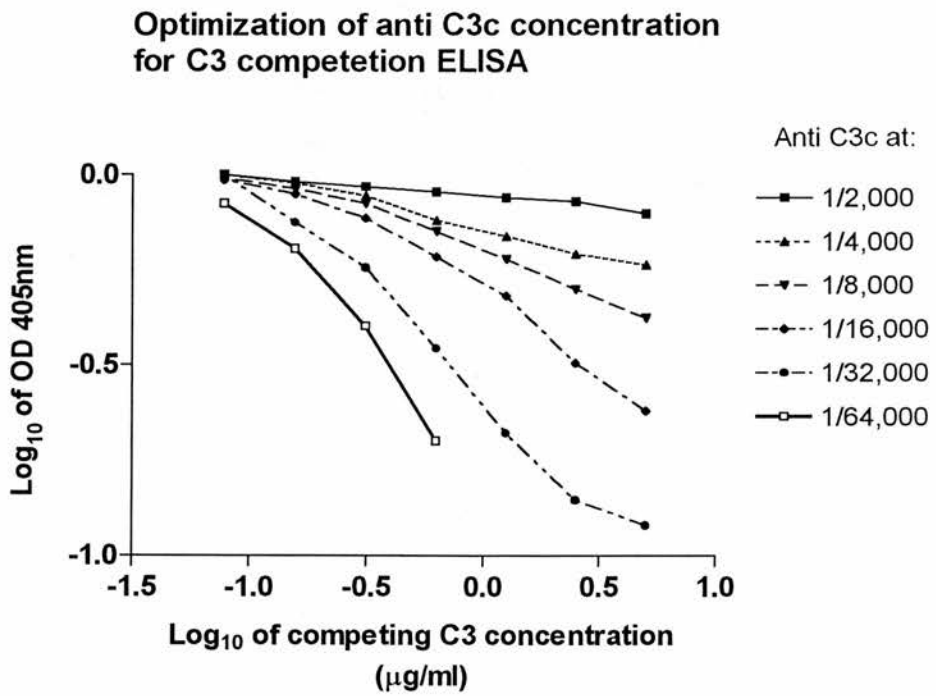

Fig 3.5: Optimization of the quantity of anti C3c required to detect competition for antibody between immobilised and free C3

An Immulon 2 ELISA plate was coated with 0.3 μg of purified C3. Between 0.25 μg and 3.9 ng of competing C3 was then added, together with anti C3c at dilutions between 1/2,000 and 1/128,000 (0.125 μg to 2 pg). Anti C3c bound to the immobilised C3 was then detected with alkaline phosphatase conjugated goat anti rabbit at 1/16,000

b) The state of the competing antigen - influence of duration of thawing

Purified C3 which had only just been thawed gave less binding of anti C3c (i.e. less competition and a higher signal due to anti C3c binding to immobilized C3 on the plate) than was the case with purified C3 kept overnight at 4°C after thawing (data not shown). This may have been because slow conversion of the C3 occurs both on the plate and in the C3 kept for use as the standard. With C3 in the form of C3c on both the plate and in the competing antigen, anti C3c reacted optimally with both.

However, if freshly thawed C3 was used as the competing antigen it may have been that the C3 is intact and since this lacked C3c, epitopes and would not compete for the anti C3c to the same degree.

3.3.2.5 Assay Performance:

WGLF C3 limit of detection (sensitivity)

C3(c) was detectable in WGLF and it's detection by anti C3c gave a parallel dose response curve to that seen with the purified C3 standard (Fig 3.6). From a response curve for the C3 standard, at concentrations lower than 0.16 μg/ml the response curve was no longer linear. Beyond this, the sensitivity of the assay reduced with increasing dilution. This therefore represented the lowest concentration which could be accurately measured from this standard curve. With a minimum testing dilution of WGLF at 1/2, this gave a lower limit of detection (sensitivity) of 0.3 μg/ml.

The recovery of purified C3 added to WGLF was generally high (>80%) except for one case where the WGLF concentration was very high in relation to the spike added. This indicated that the ELISA was valid for quantifying C3 in WGLF but that detection of high concentrations might not have been quantitative.

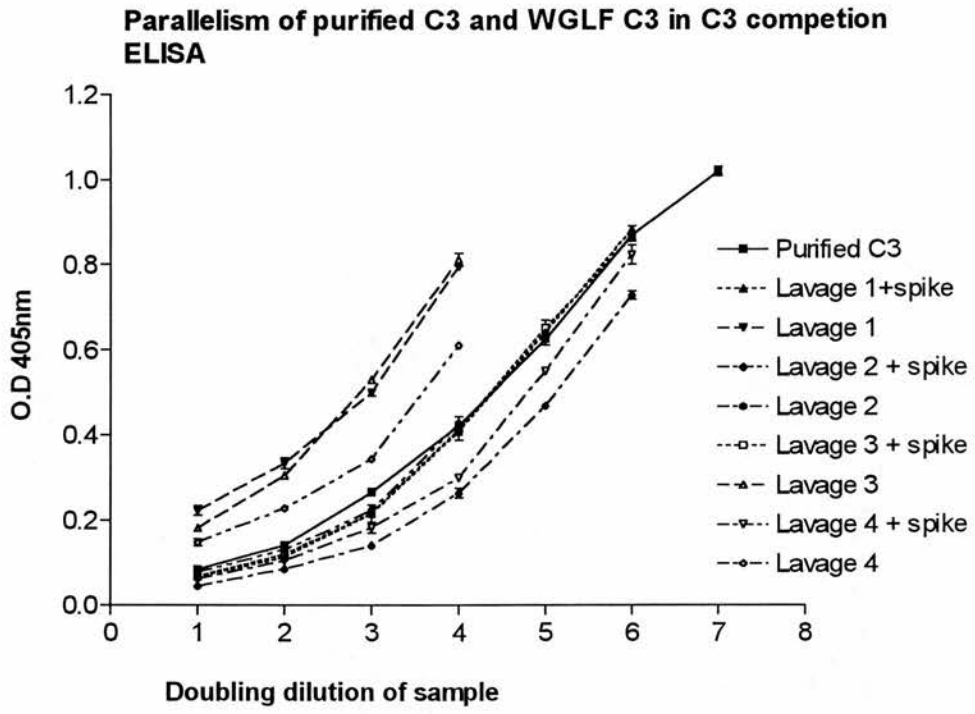

Fig 3.6: Parallelism between purified C3 and WGLF C3 in competition ELISA

Purified C3 was detected similarly if assayed alone or together with WGLF C3, indicating that the assay detected WGLF C3 in the same way as the C3 standard.

TABLE 3.2 Recovery of C3 added to WGLF (quantified by competition ELISA)

WGLF	WGLF dilution	spike µg/ml	C3 µg/ml	mean result	recovery %
1	2	N	3.2	3.4	
1	4	N	3.6		
1	8	N	3.7		
1	16	N	3.1		
1	4	1.25	3.5		167
1	8	0.625	1.6		152
1	16	0.3125	0.7		133
1	32	0.15625	0.3		114
2	4	N	11.7	11.8	
2	8	N	11.9		
2	16	N	9.9		
2	8	0.625	2.7		129
2	16	0.3125	1.2		114
2	32	0.15625	0.5		95
3	2	N	3.9	3.9	
3	4	N	4		
3	8	N	3.3		
3	16	N	3		
3	4	1.25	3.2		144
3	8	0.625	1.5		135
3	16	0.3125	0.6		108
3	32	0.15625	0.3		108
4	2	N	5	5.7	
4	4	N	5.8		
4	8	N	6.7		
4	16	N	5.5		
4	4	1.25	3.8		142
4	8	0.625	1.9		142
4	16	0.3125	1		150
4	32	0.15625	0.4		120

For each WGLF (1-4) take the unspiked (N) result for the dilution of interest and divide by the dilution factor. Then, select that dilution for the spiked WGLF. Add the concentration of the spike to that observed for the unspiked WGLF. This gives the expected result. Recovery % = actual/expected x 100. Read off the actual result for spiked WGLF from the appropriate test dilution.

The table shows that the recovery of spiked C3 in WGLF was approximately 100 % . The reduced recovery at greater test dilution may reflect the limit of sensitivity for the assay.

3.3.2.6 Influence of gut proteases on detection of C3 in WGLF

There is indirect evidence that detection of C3 in WGLF is less affected by the action of gut proteases than is albumin

TABLE 3.3 Comparison of detection of C3 with that of albumin

WGLF parameter	CDi	CDa	UCi	UCa
raised albumin with raised C3	0/13	9/13	0/13	13/14
raised C3 with normal albumin	4/13	4/13	2/13	0/14
raised albumin without raised C3	0/13	0/13	0/13	1/14

Number of cases in which WGLF C3 > 1 μg/ml and albumin >20 μg/ml (here I have assumed that if WGLF albumin is >20 μg/ml then albumin loss is raised)

If degradation of proteins during gut transit had a significant effect on the concentration of that protein in WGLF, it might have be expected to see a relationship between the protease activity of unprocessed WGLF and how much of that protein was detected in the processed WGLF. The assay for protease activity is discussed in chapter 6.

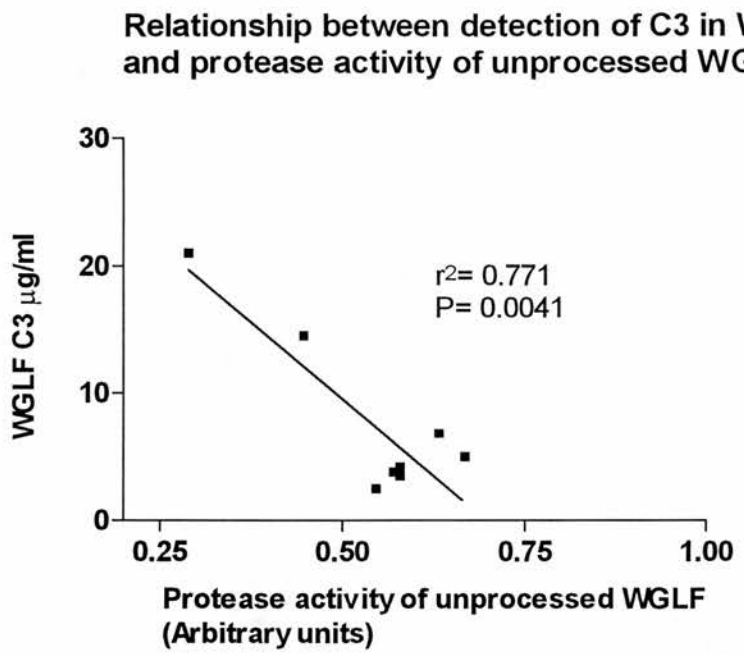

Fig 3.7: Relationship between recovery of C3 in WGLF and WGLF protease activity

C3 was detected in filtered processed WGLF as described in this chapter. Protease activity was measured in unprocessed WGLF by dye release from Azocoll as described in thesis appendix.

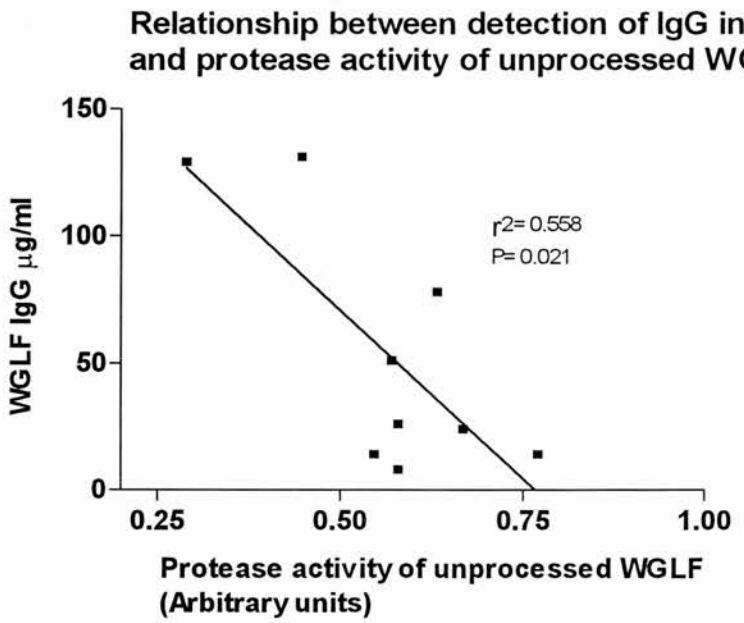

Fig 3.8: Relationship between recovery of IgG in WGLF and WGLF protease activity

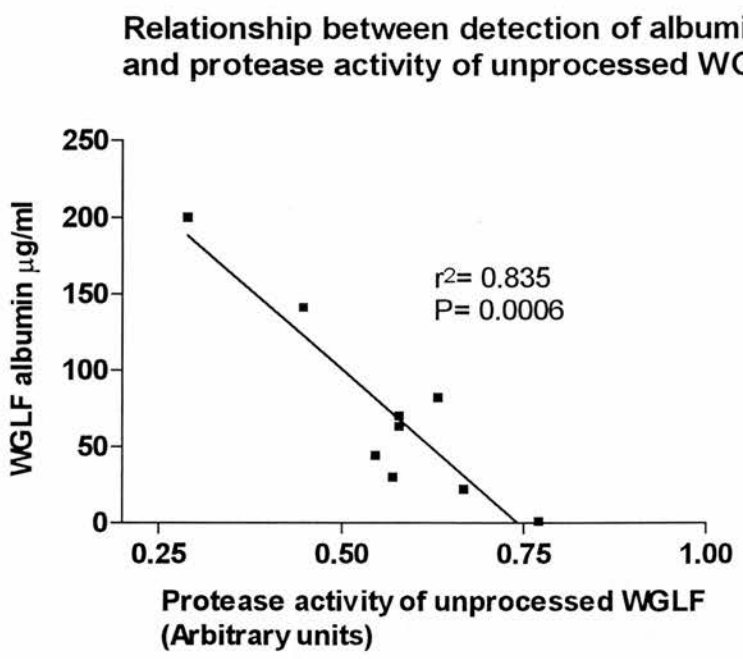

Fig 3.9: Relationship between recovery of albumin in WGLF and WGLF protease activity

WGLF from 8 patients with active IBD with raised WGLF C3 was tested for protease activity (in the unprocessed sample) using dye release from Azocoll as described in the thesis appendix. The linear regression line shows a strong negative association between C3 detected and WGLF protease activity. One patient with a spurious result, protease 0771, C3 13 µg/ml has been removed as inclusion of this data abrogated the negative association seen between WGLF C3 concentration and protease activity of unprocessed WGLF

All three proteins showed a strong negative association between the WGLF concentration of that protein and protease activity of unprocessed WGLF. This indicated that proteolysis during gut transit resulted in less of that protein being detected. As the same patients have been used for each protein, the strength of this association should give an indication as to the susceptibility of that protein to proteolysis. The results would suggest that the order of protein susceptibility to proteolysis is albumin > C3 >> IgG. This has implications in chapter 6, where the relative loss of each protein has been compared. Ideally, the effect of WGLF proteases on recovery of C3 should have been assessed directly, by measuring C3 in WGLF that has been incubated at 37°C prior to processing.

One reason why this was not done is that a competition assay has been used to quantify C3. Generation of fragments might have therefore produce greater competition for the anti C3c antibodies, overestimating C3 concentration.

3.3.2.7 Quantifying C3 in serum

Serum gave a dose response curve which was not as parallel to the standard as that for WGLF and this deviation was increased at greater dilutions of serum. If the OD readings for serum at a dilution of 1/500 were above that representing maximum competition, the test result was taken as the mean of values obtained at 1/500 and 1/1,000. If the OD at was lower than that representing maximum standard competition, the test result was taken as the mean of the results at 1/1,000 and 1/2,000. The accuracy and precision of C3 concentrations calculated for serum were therefore likely to be lower than that for WGLF.

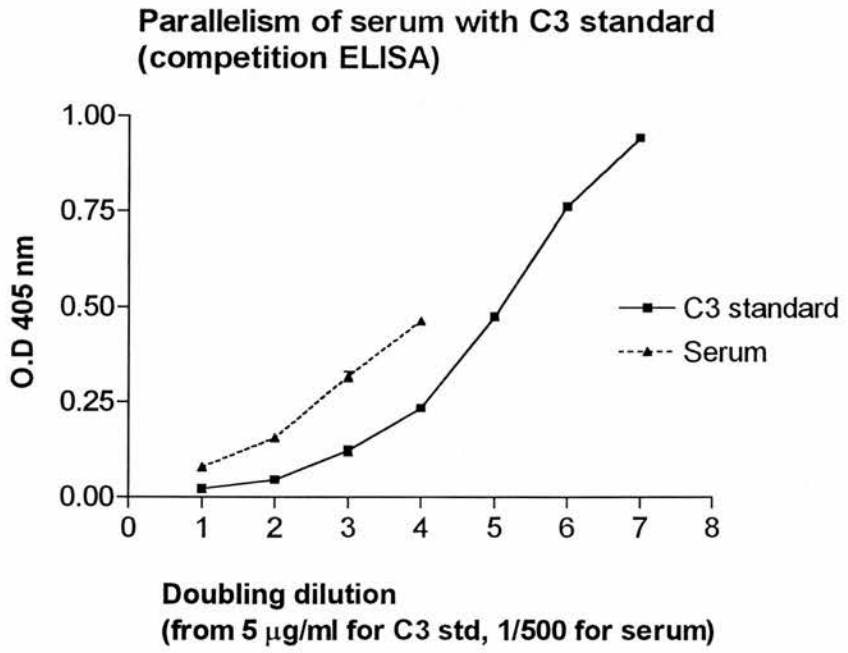

Fig 3.10: Parallelism between C3 in serum and purified C3 in competition ELISA

50 µl of serum at dilutions from at 1/500 to 1/64,000 or, purified C3 at 0.625 to 5 µg/ml were added simultaneously with 50 µl of anti C3c at a dilution of 1/32,000 to 0.3 µg of immobilised C3. The graph shows that competition for anti C3c is similar whether purified C3 or serum is used.

3.3.2.8 Assay Performance of competition ELISA for C3

a) Within batch variation (WGLF)

This gave a mean value of 4 μg/ml with a standard deviation of 0.24 giving a within batch coefficient of variation of 6%.

b) Between batch variation (WGLF)

Over 26 runs, the mean WGLF QC C3 concentration was 0.84 μg/ml, with a standard deviation of 0.14, giving a coefficient of variation of 16.7%.
Two WGLF samples (C3 21.2 and 4.2 μg/ml respectively) were analysed on three different days.. These gave a between batch standard deviation of 0.57 μg/ml and a CV of 4.5%. Therefore the assay might have been more reproducible with higher concentrations of WGLF C3.

c) Within batch variation (serum)

This gave a mean value of 1,207 μg/ml with a standard deviation of 96.3 μg/ml, giving a within batch coefficient of variation of 8%.

d) Between batch variation (serum)

$SD = \sqrt{\sum d^2/n}$ where d = difference between duplicate values and n = number of pairs. This gave a standard deviation of 313 μg/ml with a CV of 31%. This high between batch variability of serum C3 probably reflected the greater non-parallelism between serum C3 and the purified C3 standard and the difficulty in choosing the most parallel dilutions.

Final method for detection of C3 in WGLF by competition ELISA

Reagents

Coating buffer 0.05 M carbonate/bicarbonate buffer pH 9.6

Sample/blocking buffer 1% BSA in a solution of 0.05 % Tween 20 in 0.9 % saline.

C3 standard Sigma purified C3 stored at -70°C then thawed at 4°C for 16 h before use.

Anti C3c Rabbit anti human C3c (Sigma)

Anti rabbit IgG Goat anti rabbit IgG alkaline phosphatase conjugate (Sigma).

Samples WGLF filtered and processed (as described in thesis appendix)

Procedure

1. Immulon 2 plates were coated 100 µl/well with purified human C3 diluted to 3 µg/ml in coating buffer. The plate was incubated overnight at 4°C.
2. Unbound C3 was removed by washing the plate 3 x with saline/Tween.
3. Unreacted binding sites were blocked by filling the wells with sample buffer and incubating the plate for 1 h at 37°C.
4. After decanting the blocking solution, 50 µl/well of fresh sample buffer was added (100 µl to 'blank' wells).
5. C3 standard was diluted to 5 µg/ml in sample buffer and then 50 µl/well was added to duplicate wells and a doubling dilution series produced on the plate.
6. Test samples were diluted 1:1 in sample buffer before adding 50 µl/well to duplicate columns and four doubling dilutions prepared on the plate.
7. Rabbit anti human C3c was diluted to 1/32,000 in two stages (1/320 then 1/100) in sample buffer and 50 µl added to all wells except the blank. The plate was then incubated for 2 h at 37°C.
8. Unbound antibody was removed by washing the plate 3 x with saline/Tween.
9. Goat anti rabbit IgG alkaline phosphatase conjugate was diluted 1/16,000 in sample buffer and 100 µl added to all wells. The plate was incubated for 2 h at 37°C.

10. Unbound antibody was washed free as before.
11. The colour reaction with p-nitrophenol phosphate was carried out as described in the thesis appendix. The OD at 405 nm was read.
12. Data for the standards OD readings and $\log_{10}$ of C3 concentration was fitted to a one site competition equation (Graphpad Prism), giving a non-linear regression equation. Unknown test concentrations were then calculated from the OD results obtained. The final result was given as the mean value of two or more pairs of doubling dilution points which were parallel to the standard curve.

3.4 Discussion

Intact C3 is present in plasma and can be fixed. C3 was detectable over a range of concentrations, although, the 'hook' of the response curve at high concentrations may indicate that assay conditions were sub-optimal. Serum gave much lower detection of intact C3, probably reflecting gradual tick-over conversion of C3 during storage at 4°C. In plasma this was inhibited by EDTA and storage at -20°C. The finding that no fixation occured with WGLF might indicate that either there was no C3 or that C3 was spent.

It was possible to develop a competition ELISA using anti C3c that was sensitive enough to quantify C3 in WGLF. C3 in WGLF showed parallel dose response curves to that for a purified human C3 standard. This was true when purified C3 used for the standard was kept overnight at 4°C before use, as for C3 used as the coating antigen. This pre-incubation may have resulted in slow turnover of C3 to C3c. C3 in WGLF was likely to be in the form of C3c (or other activated C3 fragments). This might explain the parallelism observed under these conditions. The similar recognition of WGLF and purified C3 by anti C3c was confirmed by the high recovery of purified C3 when added to WGLF.

Between batch variability for the assay was quite high but this was not likely to result in many false positive results. The high dilution factor used for anti C3c may have been responsible for this. C3 was also detectable in serum although at low precision. The dose response curve for serum was not parallel to the standard, possibly because serum contained some intact C3 (as shown in the fixation ELISA). Anti C3c might have bound differently to intact C3 and C3c. Consequently, serum C3 results were not used for direct patient comparison.

It is likely that the quantity of C3 detected was affected by proteolysis during gut transit. The extent of proteolysis during gut transit was dependent partly on the protease activity within the intestinal contents. This was supported by the strong negative association between the concentration of C3 in WGLF and the protease activity of unprocessed WGLF.

The finding that the strength of this association was in between that observed for albumin and IgG indicated that lability of C3 due to gut proteases was between that of these two proteins. There was a worry that proteolysis of C3 produced fragments which would actually increase the quantity of C3 detected using a competition assay. This concern appeared to be unfounded and so detection of C3 reflected how much was secreted. WGLF is therefore a suitable medium in which to quantify C3 lost to the gut. The competition ELISA for C3 would later be used to compare complement loss to the gut in patients with IBD to patients with non-inflammatory intestinal diseases.

SECTION 3 RESULTS

CHAPTER FOUR

The structural nature of immunoglobulins in WGLF

4.1 WGLF immunoglobulin binding to T-Gel

The quantity bound was calculated by subtraction of amount in the supernatant (unbound) from that added. The quantity eluted is that released upon adding elution buffer.

TABLE 4.1 IgM binding to T-Gel

volume of WGLF (ml)	µg IgM added	µg unbound	µg bound	% bound	µg eluted
2 ml	16	5	11	68	14
4 ml	32	20	13	40	25
8 ml	65	42	22	35	27
16 ml	130	107	22	17	34

Different volumes of filtered processed WGLF were added to 0.35 g of T-Gel in a test tube. After mixing to allow immunoglobulin binding to occur, unbound immunoglobulins were collected in the supernatant following centrifugation. After washing, the T-Gel was equilibrated with eluting buffer and the released immunoglobulins collected. Immunoglobulin content was assayed by sandwich ELISA. IgM bound = what is added - what remains in the supernatant. The % bound = this quantity/ what is added x 100. Quantity added = WGLF concentration (µg/ml) x volume (ml) added to T-Gel.

The results for IgM binding demonstrate two effects: Firstly, not even at the lowest loading did all of the IgM binds. Furthermore, the % of IgM which bound drops as the quantity of IgM added was increased. This indicated that binding sites on the T-Gel resin are limiting. However, the finding that more IgM binds the more is added would suggest that there was competition for binding sites.

One speculative explanation to as why not all the IgM bound equivalently to the T-Gel equivalently could be if some WGLF IgM molecules were partially degraded by gut proteases. The finding that more IgM was eluted than was calculated to have bound to the T-Gel could be due to partial degradation of IgM during the experiment with subsequent detection of immunoglobulin fragments.

TABLE 4.2 IgA binding to T-Gel

Volume of WGLF (ml)	μg IgA added	μg unbound	μg bound	% bound	μg eluted
2 ml	104	30	74	71	38
4 ml	208	95	113	54	67
8 ml	416	252	164.	39	102
16 ml	832	693	139	17	118

TABLE 4.3 IgG binding to T-Gel

Volume of WGLF (ml)	μg IgG added	μg unbound	μg bound	% bound	μg eluted
2 ml	25	9	16	65	6
4 ml	50	27	22	45	10
8 ml	99	74	26	26	14
15.5 ml	198	178	21	11	ND

Optimal immunoglobulin binding of between 60 and 70% was obtained with a loading of 2 ml of WGLF/ 0.37 g T-Gel. Given a total WGLF immunoglobulin content of 75 μg/ml this equated to a loading of 405 μg immunoglobulin/g of T-Gel. It was decided to use this loading ratio for chromatography on the premise that binding might be expected to be more efficient in a column.

Elution of bound immunoglobulins from T-Gel in a test tube was poor. This might be expected to be improved in a column as eluted protein cannot then re-encounter the T-Gel ligand. The result of more IgM recovered from T-Gel than was loaded suggested IgM fragmentation.

4.2 T-Gel chromatography

A 30 ml (22.2 g of dry T-Gel) was used.

4.2.1 Definitions of the various column fractions

Protein passing through the column during sample addition is LOADING fraction.
Protein released with equilibrating buffer is the WASH fraction.
Protein released with the eluting buffer is the ELUTE fraction.

4.2.1.1 T-Gel chromatography run 1 (WGLF)

100 ml of WGLF (total immunoglobulin content of 90 μg/ml) was added to a column of 22.2 g T-Gel. From the results of the test tube study, this was predicted to be equivalent to 65 % of the maximum immunoglobulin binding capacity. A flow rate of 30 ml/h rather than the original 48 ml/h was used for loading. This was to improve the binding efficiency from WGLF which has a low immunoglobulin concentration compared to media used for testing the T-Gel.

In this experiment WGLF corresponding to a total IgA, IgG and IgM of 9,180 μg was added to 22 g of T-Gel, i.e., 417 μg of immunoglobulin per gram of T-Gel. This is similar to when 2 ml of WGLF, corresponding to a total of 145 μg of IgA, IgG and IgM was added to 0.35 g of T-Gel (414 μg/g) in the test tube study. The % of WGLF immunoglobulins binding to the T-Gel using a column was lower at an average of 49 % than the 68 % seen in the test tube study. Immunoglobulin binding to T-Gel in a column was less than predicted and it may be necessary to reduce column loading to give maximum binding. Maximization of immunoglobulin binding was necessary to allow inference that non-binding immunoglobulins were not be structurally intact.

TABLE 4.4 T-Gel chromatography run 1, WGLF

Chromatography stage	Volume (ml)	Flow rate ml/h	μg IgA	μg IgG	μg IgM
WGLF	100		4,030	4,180	970
Loading fraction	100	30	1,340	270	450
Wash	100	30	946	1,715	65
Elute (overnight)	96	6	2,608	1,910	364
50 % ethylene glycol	60	60	233	45	20
Total recovered			5,125	3,940	900
% of added immunoglobulin which binds to T-Gel			55	50	43

WGLF was added to a T-Gel column (22 g of T-Gel). Fractionated unbound material was collected by washing the column. Bound material was eluted by changing the buffer. Theoretically, the quantity of immunoglobulin bound = that added -(that in the loading fraction + that in the wash fraction. Eluted immunoglobulin = that released with elute + that released with ethylene glycol. Recovered immunoglobulin = sum of all column fractions. The % bound = (bound/added) x100.

For IgA and IgG, a similar fraction of the added immunoglobulin bind to the T-Gel, supporting the finding seen with the test tube binding study. However, the Immunoglobulin recovery of greater than 100% may indicate immunoglobulin degradation during the experiment. This may have been because chromatography was run overnight at room temperature. It may be beneficial to run the column at 4°C to preserve immunoglobulin structure.

It appeared that immunoglobulin recovery was greater than 100 % for IgA. This implied that IgA is less stable than IgG or IgM, in contrast to previous findings (Brown et al, 1970). It may alternatively have been that fragments of IgM and IgG were rapidly degraded and not detectable by ELISA.

As WGLF used in this experiment was rich in IgA, IgG and IgM, it was decided to investigate the structural nature of these immunoglobulins.

WGLF immunoglobulin from the loading, wash and elution stages were subjected to SDS-PAGE (described in the methods section).

TABLE 4.5 Quantity of WGLF immunoglobulins used for electrophoresis

Chromatography stage	IgA μg/ml	IgA μg	IgG μg/ml	IgG μg	IgM μg/ml	IgM μg
WGLF	40	2.0	42	2.1	10	0.5
throughput	30	3.0	10	1	12	1.2
wash	16	1.6	28	2.8	1	0.1
elute	27	2.7	39	3.9	9	0.9
ethylene glycol	17	1.7	3	0.3	1	0.1

The quantity of immunoglobulin used in the electrophoresis was calculated from:
a) Concentration in fraction pool.
b) x concentration factor (5 for WGLF, 10 for column fraction).
c) x 2/3 (to account for dilution by reducing agent).
d) x 30/1,000 (the volume loaded on the gel).

Detection of specific antibodies in T-Gel fractions

The detection of specific antibodies of a particular class requires an intact antigen binding site associated with part of the heavy chain. This can be used to investigate whether WGLF immunoglobulins binding to the T-Gel are more intact than those that do not, i.e. are the binding immunoglobulins enriched for specific antibody ?

ELISA for antibodies against a cocktail of LPS core antigen (EndoCAb) from luminal bacteria; E.Coli, Klebsiella,(Barclay, G.R. and Scott, B.B. 1987) was performed by Dr Samiul Hoque. The method is outlined in the thesis appendix.

TABLE 4.6 antibodies to endotoxin (EndoCAb) T-Gel run 1, WGLF

a) IgG

Chromatography stage	EndoCab IgG arbitrary units	IgG units /mg IgG	Total protein mg/ml	IgG units /mg protein
WGLF	0.191	4.6	2.51	0.08
Loading fraction	0.009	6.9	1.50	0.01
Wash	0.004	0.8	0.44	0.01
Elute	0.092	4.9	0.31	0.30
Ethylene glycol	0.027	27.5	0.06	0.45

WGLF and T-Gel fractions were assayed by ELISA for IgG antibodies to endotoxin core antigen as described in the thesis appendix. The number of arbitrary units is low because a serum reference standard has been used and the antigen is likely to be encountered via a mucosal rather than systemic route.

b) IgA antibodies to endotoxin (EndoCab) T-Gel run 1, WGLF

Chromatography stage	EndoCab IgA arbitrary units	IgA units /mg IgA	IgA units /mg protein
WGLF	1.26	31.3	0.50
Loading fraction	0.357	26.6	0.24
Wash	0.251	31.4	0.50
Elute	0.638	24.7	2.13
Ethylene glycol	0.058	14.1	0.97

WGLF contained more IgA than IgG EndoCab units, reflecting a mucosal IgA immune response. The finding that IgA endoCab units expressed per mg of IgA did not show enrichment in the T-Gel binding fractions might reflect the possibility that IgA against this antigen forms only a small part of the total mucosal IgA response. EndoCab specific IgA were just as likely to be intact or degraded as any other IgA. However, when EndoCab IgA is compared to WGLF total protein, it is clear that detection of specific IgA is enriched in the T-Gel binding fractions. This suggested that T-Gel binding immunoglobulins are more intact than non-binding immunoglobulins. Later this finding was verified by SDS-PAGE.

Chromatography profile for IgA

Chromatography gave a clean separation of binding and non-binding immunoglobulins (Fig 4.1) Notice that elution of bound protein began after 30 ml of eluting buffer has been passed. This represented the void volume of the column.

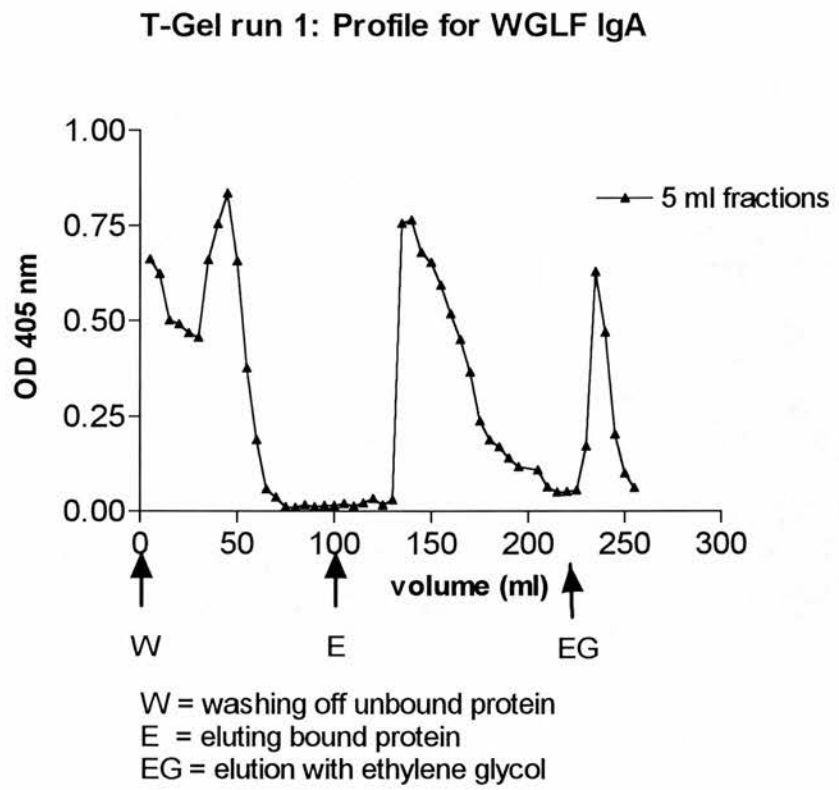

Fig 4.1: Chromatography profile for WGLF IgA using T-Gel chromatography
Graph represents T-Gel experiment 4.1

4.2.1.2 T-Gel chromatography run 2 (serum)

The quantity of immunoglobulins binding to the T-Gel using WGLF were much lower than 65 mg/g of T-Gel observed in the original work.
To test whether this might be because of the dilute nature of WGLF, it was decided to run an experiment using serum. An even slower flow rate was used as it was felt that the faster flow rate had been detrimental to binding of WGLF immunoglobulins.

TABLE 4.7 T-Gel run 2, serum

Chromatography stage	Volume (ml)	Flow rate ml/h	IgM μg	IgA μg
Serum added	4	12	8,000	6,800
Wash	110	18	460	380
Elute	80	90	3,050	1,860
50% Ethylene glycol	80	90	0	0
Unaccounted for			4,490	4,560
% Bound *			94	94

Serum was added to a column of 22 g fresh T-Gel. After extensive washing, bound immunoglobulins were released using a two stage elution process. As the sample volume was less than that of the column, the wash fraction comprises initially non-binding material in addition to that released during washing. Unaccounted for immunoglobulin = added -(wash + elute + ethylene glycol)

Much of the added immunoglobulins are unaccounted for after an extensive elution regimen. It has been assumed that unrecovered immunoglobulin remains bound to the T-Gel after elution. The other possibility that immunoglobulins could be degraded during that experiment was unlikely because chromatography was conducted in a cold room and more rapidly than in run 1. The % bound has been calculated from: [(elute + unaccounted for)/added] x 100. Both the efficiency (%) and quantity of immunoglobulins binding were much higher than observed with WGLF.

Possible reasons for this could have been because WGLF is very dilute. WGLF contains polyethylene glycol (PEG) which may interefere with immunoglobulin binding as ethylene glycol, a similar chemical, has been used to enhance elution of bound immunoglobulins from T-Gel.

4.2.1.3 T-Gel chromatography run 3 (serum diluted in PEG)

An aliquot of the serum pool used for the previous experiment was diluted 100 fold in polyethylene glycol 3450 solution (final concentration 4.7 g/100 ml as for WGLF). This would show whether the low binding of WGLF immunoglobulins was due to the PEG. T-Gel was recycled and the column repacked for this experiment.

TABLE 4.8 T-Gel run 3, serum diluted in PEG

Chromatography stage	Volume ml	Flow rate ml/h	IgA μg	IgM μg	IgG μg
Serum added	100	6	1,700	2,000	14,500
Loading fraction	100		580	290	6,000
Wash	84	40	220	180	410
Elute	70	40	250	280	3,590
50 % Ethylene glycol)	70	40	130	410	1,110
unaccounted for			520	840	3,390
% bound *			53	77	56

Serum diluted in polyethylene glycol was added to a column of 22 g T-Gel. After washing, bound immunoglobulins were eluted using a two stage procedure. Unaccounted for immunoglobulin has been assumed to remain bound to the T-Gel following elution.

Despite a reduced immunoglobulin loading, the % of added immunoglobulins which bind to the T-Gel was reduced. It is likely that the addition of PEG was responsible. The low % of binding in WGLF might have been because PEG interferes with the interaction between immunoglobulins and the T-Gel.

To override this and allow maximal binding of WGLF immunoglobulins it was necessary to reduce column loading. Binding efficiency for serum immunoglobulins diluted in PEG was slightly better than that observed for WGLF, possibly as a result of slower column loading.

4.2.1.4 T-Gel chromatography run 4 (WGLF, cold room)

The column was cleaned, fitted with a new bed support and packed with 17 g fresh T-Gel at 4°C, giving a bed volume of 23 ml. Chromatography was performed in a cold room to minimize possible degradation of WGLF immunoglobulins (apparent from the first experiment run at room temperature, 21°C).
Reduced immunoglobulin loading was coupled with slow loading rates to try to maximize binding. Elution was performed using reversed flow to enhance elution efficiency (advice from Pharmacia).

TABLE 4.9 T-Gel run 4, WGLF in cold room

Chromatography stage	Volume (ml)	Flow rate ml/h	IgA μg	IgA μg/ml	Ovalbumin specific IgA units/mg IgA
WGLF	13.5	10	1,598	118	5.5
Wash	58.8	10	131		
Elute	58.8	10	853		
Elute (peak fraction)	4.2			124	6.1
Ethylene glycol	25.2	10	0		
Unaccounted for			614		
% bound *			92 %		

* Assuming unaccounted for IgA remains bound to the column following elution. Individual fractions of eluted material were assayed for total and specific IgA. Data for IgG and IgM is not included as concentrations of these were very low.

The percentage of WGLF IgA binding to the T-Gel was much greater than in run 1. It would appear that a reduction in immunoglobulin loading combined with slow flow rate has improved the efficiency of immunoglobulin binding.

The efficiency of elution [eluted -(added -wash)]/100 is 58 % with an IgA loading of 94 μg/g of T-Gel. This is similar to the 51 % seen in the test tube binding study with an IgA loading of 297 μg/g T-Gel. The inability to release all of the bound immunoglobulin was therefore likely to reflect sub-optimal performance of the T-Gel.

IgA antibodies to ovalbumin were assayed by ELISA (method in thesis appendix) to assess whether T-Gel binding IgA was enriched for specific antibodies and hence more intact than non-binding IgA. There was approximately 10 % more anti ovalbumin IgA in the T-gel binding IgA then in the WGLF IgA. This agreed with approximately 90 % of WGLF IgA binding to the T-Gel (assuming that binding was optimal).

4.2.1.5 T-Gel chromatography run 5 (unfrozen WGLF, cold room)

T-gel was re-cycled and the column re-packed at 4°C. WGLF was loaded at a similar loading to the previous experiment to test whether the high % binding achieved was reproducible. The only difference was that the WGLF was used directly after processing i.e. it had not been frozen and thawed.

TABLE 4.10 T-Gel run 5, unfrozen WGLF in cold room

Chromatography stage	Volume (ml)	Flow rate ml/h	IgA μg	protein mg/ml	IgA/ mg protein
WGLF	19	18	920	2.06	23.5
Wash 1	15	36	45	2.19	1.4
Wash 2	15	36	90	0.55	10.9
Elute 1	15	36	90	0.19	31.6
Elute 2	15	36	386	0.35	73.5
Ethylene glycol	20	36	308	0.24	64.1
% Bound			85 %		

Data for IgG and IgM is not included as these were at very low concentration.

Specific binding of WGLF IgA to the T-Gel is demonstrated by the enrichment in IgA/mg of protein in the eluted fractions. As in the previous experiment, a high % of added IgA bound to the T-Gel.

As the IgA loading was reduced for this experiment, it may have been expected to see a greater % of added IgA binding. The finding that this had not changed indicated that binding of WGLF IgA was now maximal and so IgA not binding to the T-Gel may be unable to do so. Results for antigen specific antibodies in the previous experiment may indicate that this IgA was fragmented.

Unfortunately, ovalbumin specific IgA were undetectable in WGLF used for the current experiment. It is of interest to note that the elution efficiency was much higher in this compared to any of the other experiments. As the only difference was that the sample had not been frozen and thawed inference might be made that freezing and thawing immunoglobulins alters their structural nature in a manner which changes their interaction with the T-Gel ligand. It must be assumed that the original T-Gel work was done using unfrozen specimens.

4.3 Electrophoresis

Migration of the molecular weight markers was proportional to the $\log_{10}$ of their molecular weight (Fig 4.2), validating the estimation of molecular weight of unknown proteins by comparison of their migration to that of the markers.

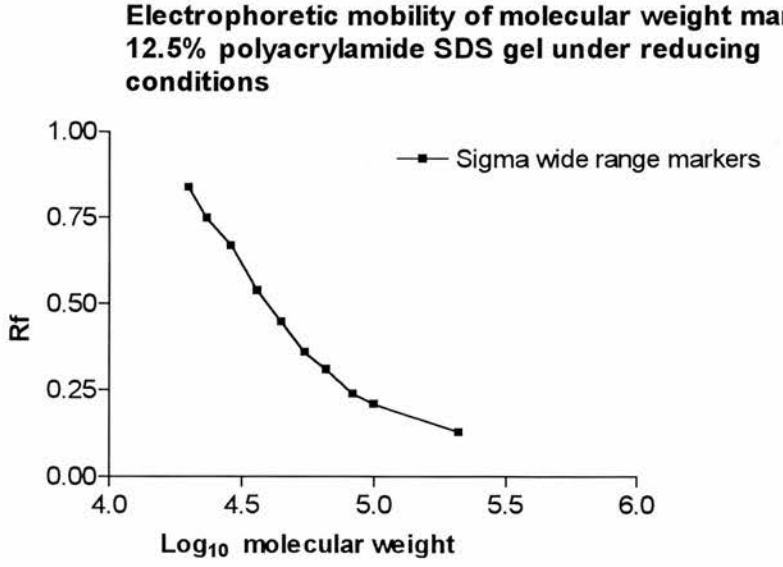

Fig 4.2: Relationship between electrophoretic migration and protein molecular weight using SDS-PAGE

A 12.5 % discontinuous gel was loaded with 5 µl of Sigma wide range molecular weight markers and electrophoresis performed as described in the thesis appendix.

4.3.1 Gel staining of T-Gel fractionated WGLF proteins

Coomassie blue staining of T-Gel separated proteins was faint and sharp, indicating low protein concentration but good separation. It was not possible to obtain a photographic reproduction because of the faint staining. Observation at the time revealed the following pattern:

LOADING fraction:

major bands at 66 Kd (probably albumin)

faint bands at 55 Kd and 25 Kd (probably heavy and light immunoglobulin chains).

fuzzy staining at just less than 55 Kd (several proteins of similar mass).

Elute:

bright staining at 55 Kd and 25 Kd

very faint 40 Kd band

This suggests that immunoglobulin is the major protein binding to the T gel.

4.3.2 Detection system for WGLF IgA on Western blot

TABLE 4.11 a) Single antibody system for detecting IgA

Antibody dilution	Time of incubation	Wash	Substrate	Result
1/500	1 h	4 x 2 min TBS-Tween	BCIP/NBT	all spots bright, high background
1/1,000	1 h	"	"	"
1/2,500	1 h	"	"	bright spots, low background
1/500	2 h	"	"	bright spots, high background
1/1,000	2 h	"	"	"
1/2,500	2 h		"	"

One microlitre of colostrum IgA spotted onto nitrocellulose was detected using goat anti human IgA alkaline phosphatase conjugate. All volumes 1 ml, with antibody dilutions in TBS-Tween.

TABLE 4.12 b) Two antibody system for detecting IgA

Primary antibody	Wash	Secondary antibody	Wash	Substrate	Result
1/500		1/1,000		NBT/BCIP	bright spots, high background
1/1,000		"		"	bright spots, some background
1/2,500		"		"	bright spots, low background
1/2,500		control no 2nd Ab		"	no staining
control no 1st Ab		1/1,000		"	weak spot staining

One microlitre of colostrum IgA was detected with anti alpha chain antisera followed by anti species alkaline phosphatase conjugate. All antibody incubation periods were one hour at room temperature.

Non specific binding of the anti goat Fc to human IgA makes the two antibody system unsuitable. The single antibody system gave clear staining with low background when using anti α chain at 1/2,500 incubated for one hour.

4.3.3 Staining of Western blot

a) General protein staining

Ponceau S gave a clear pink stain for transferred proteins. No staining was evident at the low molecular weight end of the blot.

b) Antibody staining of immunoglobulin heavy chain

IgG developed quickest, then IgM but, IgA hardly developed at all. Heavy chain staining at a band at 55 Kd was more intense and compact with the T-Gel binding fraction compared to the WGLF loading fraction. IgG staining gave three bands at greater than heavy chain molecular weight and two bands with lower molecular weight, in addition to the major band at 55 Kd. IgM only gave staining at 60 Kd.

L = Loading fraction (WGLF)
U = Unbound fraction
W = Released by washing
EL = Elute

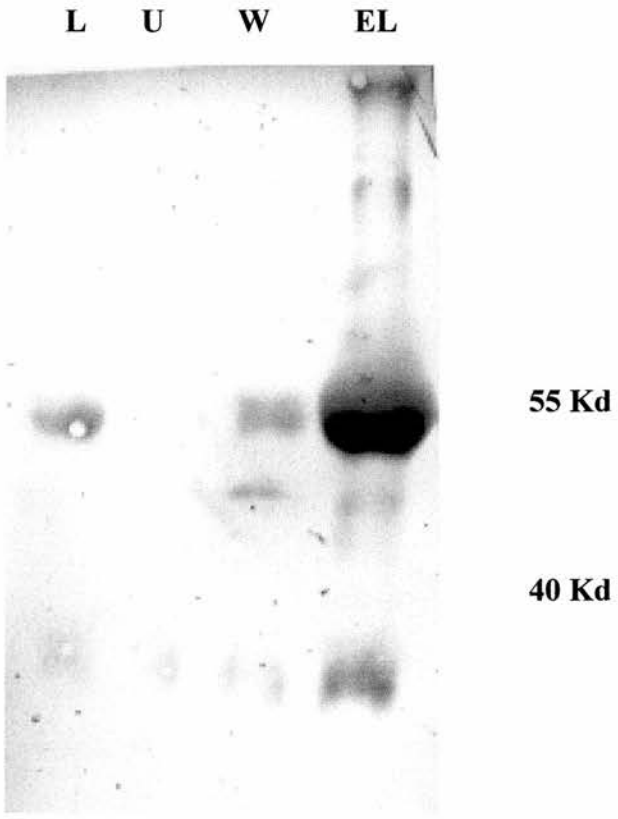

Fig 4.3: Western blot from T-Gel run 1, stained with anti γ chain

Anti γ chain staining of the 55 Kd heavy chain band was several fold more intense with the T-Gel binding fraction than with unfractionated WGLF. This difference in staining intensity was greater than might have been explained from the difference in gel loading. Intact heavy chain might therefore be enriched in the T-Gel binding fraction., demonstrating that the T-Gel did bind mainly intact immunoglobulins. Unfractionated WGLF gave weaker heavy chain staining and this might have reflected the presence of immunoglobulin fragments.

4.4 Discussion

We have previously shown that the recovery of immunoglobulins from faeces is very variable and dependent on the gut transit time (Ferguson et al, 1995). The whole gut lavage procedure reduces and standardizes the transit time. One advantage of this is that immunoglobulins was improved and less likely to show variation between patients. Rapid processing of WGLF with protease inhibitors reduces further degradation of immunoglobulins.

The assumption that immunoglobulins in WGLF are intact needed to be tested to validate the assays used to quantify them. Sandwich ELISA using anti heavy chain antisera detected heavy chains of both fragmented and intact immunoglobulins. Using size exclusion chromatography it was shown that α chain was detected in a wide range of molecular weight fractions from WGLF (L.McLintock, abstract: society for mucosal immunology, San Diego 1995). Immunoglobulin fragments could have given disproportionate binding of anti heavy chain as epitopes which are sterically inaccessible on the intact molecule might be revealed in the fragment. If immunoglobulins were extensively fragmented in WGLF, immunoglobulins might be been overestimated using sandwich ELISA.

Thiophilic absorption chromatography, a technique with selectivity for intact immunoglobulins was selected to assess whether WGLF immunoglobulins are intact or not. When chromatography conditions were optimized this method bound nearly all WGLF immunoglobulin. Electrophoresis was used to study the structural nature of fractionated WGLF proteins. Western blots probed with anti immunoglobulin heavy chain, demonstrated brighter staining of 55 kd IgG heavy chain in the T-Gel binding fraction than with unfractionated WGLF immunoglobulins. This confirms previous findings that the thiophilic resin selectively binds intact immunoglobulins (Hutchens et al, 1989). Non T-Gel binding immunoglobulins which were detectable by ELISA were not detectable on western blot indicating that these proteins might have been fragmented. Comparison of the relative quantity of immunoglobulin which bound or did not bind to the T-Gel has provided a useful means of assessing to what extent WGLF immunoglobulins were intact.

Further evidence for the intact nature of T-Gel binding immunoglobulins came from the finding that antigen specific antibodies were slightly enriched in this fraction when compared to unfractionated WGLF. Antigen specific antibodies were undetectable in the non-binding fraction providing further evidence that these immunoglobulins were fragmented.

It was found that under optimal conditions, 85 % of WGLF immunoglobulins bound to the T-Gel. This figure was supported by the degree of enrichment of antigen specific antibodies detected in the T-Gel binding fraction. Processed WGLF would appear to be a source of mostly intact intestinal antibodies and might be a useful medium for further studies of intestinal immunity.

It may have been useful to repeat the electrophoresis work because there was evidence of immunoglobulin fragmentation during the course of the experiment, probably because chromatography was run at room temperature. IgG staining of the western blot for T-Gel binding WGLF immunoglobulins gave, in addition to the 55 kd heavy chain, a 40 kd band that might represent a Fab or Fc fragment. In unfractionated (which had been assayed directly after thawing) this band was very weak, indicating that fragmentation of T-Gel binding immunoglobulins may have occurred. Further evidence to support this came from the finding that T-Gel binding immunoglobulins were enriched for antigen specific antibodies when compared to total protein but not when compared to total immunoglobulin. The immunoglobulin fragments were detected by ELISA.

Stability work on WGLF immunoglobulins shows that detection by ELISA is reduced with time in contact with gut proteases (chapter 6). This would suggest that immunoglobulin fragments are either not detectable or, they are broken down rapidly by proteases. Processed WGLF might have been fragmented at a slower rate and fragments remain detectable.

The western blot work gave poor binding of anti α chain. This was unexpected as 4 μg of IgA had been loaded on the gel and direct staining of a dot blot had shown 0.02 μg of IgA to be readily detectable). In a subsequent dot blot test, good binding of anti α chain was seen to unreduced IgA but poor binding to reduced IgA.

This explains why IgA was undetectable on the western blot. Anti μ heavy chain staining of T-Gel binding WGLF immunoglobulins gave a single major band at 60 Kd (not shown). This fitted with IgM having an extra domain in the heavy chain.

T-Gel ligand binds WGLF immunoglobulins of IgG, IgA and IgM classes. In a binding study, the % of WGLF immunoglobulin binding was greatest for IgA > IgG > IgM. This difference in binding efficiency might have reflected differences in the structural integrity of the three immunoglobulin classes. This would support studies which have demonstrated intestinal IgA to be more resistant to proteolysis by intestinal enzymes than is serum IgG e.g., (Brown et al, 1970).

The quantity of WGLF immunoglobulins which bound to the T-Gel was much lower than the quoted immunoglobulin binding capacity. There were several possible reasons for this:

1) Processing of WGLF includes addition of bovine serum. This contains immunoglobulins that may have competed for T-Gel binding sites but would not have been detected by ELISA's for human immunoglobulins.
This accounts for a small part of the shortfall as calculated:

a) Processing added 0.6 ml of bovine serum per 12.5 ml of processed WGLF i.e. 0.048 ml/ml.

b) As adult bovine serum contains a total immunoglobulin content of 25 g/l, this contributed 25 x 0.048 mg of immunoglobulin mg per ml of WGLF i.e. 1.2 mg.

c) 0.37 g of T-Gel should bind 24 mg of immunoglobulin (from the quoted capacity of 65 mg of immunoglobulins/ g of T-Gel). In fact only 70 % binding of 0.15 mg of human immunoglobulin was seen with a loading including 2.4 mg of bovine immunoglobulin.

2) In the original optimization experiments, γ-globulins were used as the source of immunoglobulins i.e. this was relatively pure compared with biological mixtures of protein which might have shown immunoglobulin-protein interactions that inhibited T-Gel-immunoglobulin interactions

3) Binding capacity was less with low concentrations of immunoglobulin (<2 mg/ml) (Hutchens and Porath, 1986).

Binding efficiency of WGLF immunoglobulins to T-Gel was inversely proportional to the immunoglobulin loading, indicating a limited number of ligands. This might imply that the T-Gel ligand was not synthesized correctly: elemental analysis apparatus was unavailable and so it was not possible to test this. However, T-Gel bound more immunoglobulins from serum, both in quantity and percentage of that added. It may have been worthwhile to test the immunoglobulin binding capacity using serum to see if this compared more closely with the quoted figures.

As the efficiency of binding appeared to be lower for WGLF immunoglobulins, possible reasons for this were investigated. When serum was diluted in PEG so as to resemble WGLF, the binding efficiency for serum immunoglobulins was reduced to that seen with WGLF. It might be inferred that PEG interferes with the interaction between immunoglobulins and the T-Gel ligand. PEG is likely to form hydrogen bonds with protein molecules in the same way that water molecules do. This increases the number of interactions that must be broken to allow protein interaction with the T-Gel ligand. This may have reduced the effective binding capacity of the T-Gel. There could be another factor reducing T-Gel binding of WGLF immunoglobulins as the quantity of PEG diluted serum immunoglobulins to give the same binding efficienct as for WGLF was two fold that in the WGLF tested.

When a low quantity of WGLF immunoglobulins was used, the binding efficiency was not increased on halving the quantity of immunoglobulins loaded. This would indicate that the quantity WGLF immunglobulins loaded was less than the column capacity. Unbound imunoglobulins may have been fragmented and unable to bind to the T-Gel ligand.

It was found that a large fraction of added WGLF or serum immunoglobulins were not eluted effectively from the T-Gel. This did not agree with the 95 % recovery quoted (Hutchens and Porath, 1986) and was surprising in that I had followed their conditions. I have assumed that unrecovered immunoglobulin does remain strongly bound to the T-Gel and calculations of immunoglobulin binding have taken this into account. The finding that immunoglobulin elution from the T-gel was much improved using unfrozen WGLF may indicate that freezing and thawing of WGLF was deleterious to immunglobulin structure.

These findings have demonstrated that most of the heavy chain detected in WGLF represented intact immunoglobulins and so gross overestimation of total immunoglobulin by ELISA was unlikely. This also validates the use of capture ELISA to quantify antigen specific antibodies in WGLF, opening the doors for further study of intestinal immunity. Dr Samiul Hoque at the GI unit has shown that a high proportion of healthy volunteers from Edinburgh have detectable intestinal immune responses to foods. In contrast, subjects in Bangladesh had strong intestinal immune responses to intestinal bacteria but few people had antibodies to foods. This may have reflected that improved sanitation in the west resulted in a less active intestinal immune response against bacteria, but immune responses might have occured against antigens where tolerance would be expected (Ph.D. thesis submitted 1999, University of Edinburgh,).

CHAPTER FIVE

C3 loss to the gut in inflammatory bowel disease

5.1 Introduction

Complement is important for normal clearance of antigens and immune complexes from tissues (Reviewed by Thornton et al, 1996). Activation of complement involves an amplification cascade which enhances interaction of antigen with the immune system. However, at sites where there is a large antigen load, i.e. the intestine, this could result in inflammation with damage to surrounding tissues. As C3 is pivotal to amplification of the complement cascade, it was decided to measure C3 in the gut and relate this to the presence of intestinal inflammation.

5.2 Methods

5.2.1 Patients

From a review of the gastro-intestinal laboratory database, 93 cases who had undergone whole gut lavage as bowel preparation for further investigation were retrospectively selected. 53 of these cases were confirmed cases of IBD, 12 were patients diagnosed with non IBD intestinal diseases with an inflammatory component and a further 28 were patients diagnosed as having non-inflammatory intestinal disorders or, with intestinal symptoms which were of a non inflammatory nature. This latter population was considered as a non-inflammatory control group. A group of 8 healthy volunteer individuals with no abnormal intestinal symptoms underwent whole gut lavage to provide a baseline control group.

5.2.1.1 Definition of active and inactive disease

In development of the gut lavage technique for biochemical assessment of intestinal function, a retrospectively cohort of 63 individuals comprising healthy volunteers and hospital patients under investigation of non-immunological intestinal problems was selected.

WGLF proteins were analysed as described in the thesis appendix and 'normal' reference ranges were selected on the basis of excluding values above the 95th percentile. These ranges were assigned as unlikely to represent intestinal inflammation and were as follows: IgG 0-10 μg/ml, α1AT (alpha-1-antitrypsin) 0-19 μg/ml, haemoglobin 1-5 μg/ml and albumin 0-26 μg/ml (O'Mahoney et al, 1991). In a later prospective study, IgG was quantified in WGLF from 53 well characterized patients, 35 with CD and 18 with UC. For both groups of patients clinical indices were assessed at the time of gut lavage. WGLF was then correlated with either the CDAI or the Powell-Tuck index. It was found that patients with raised indices generally had raised WGLF IgG and statistically significant correlation was seen between clinical index value and WGLF IgG in those patients with a raised index (Choudari et al, 1993). Raised WGLF IgG is therefore likely to reflect active intestinal inflammation.

Most patients without immunological abnormality of the intestine had low WGLF below 5 μg/ml. It may be that the restriction of patients used in the disease control group was not tight enough and included some patients who lose plasma proteins to the gut via mechanisms other than active inflammatory processes. For the sake of this study, I have designated patients with IBD with WGLF IgG of 7 μg/ml or greater as having active disease, using the proviso that there should be evidence of plasma leakage to the gut (raised WGLF α1AT or albumin).

Patients with Crohn's disease

Inactive = 13

4 females, mean age 48.5 yrs, SD 12.5

9 males mean age 46.3 yrs, SD 17.3

Active = 13

8 females mean age 35.9 yrs, SD 12.2

5 males mean age 38.6 yrs, SD 12.3

One male is included in both the active and inactive CD groups. He underwent whole gut lavage on two separate occasions in between which his disease activity had changed.

Patients with Ulcerative Colitis

Inactive = 13

6 females mean age 58.7 yrs, SD 11.3

7 males mean age 47.4 yrs, SD 14.1

Active = 14

5 females mean age 29.6 yrs, SD 20.2

9 males mean age 51 yrs, SD 10.7

Non inflammatory GI diseases

28 patients

19 females mean age 50.5 yrs, SD 13.5

9 males mean age 41.1 yrs, SD 13.9

see results table for diagnosis of these patients

Non-IBD intestinal diseases with inflammatory component

12 patients

6 females mean age 65.8 yrs, SD 19.9

6 males mean age 66.3 yrs, SD 12.7

Healthy volunteers

Control WGLF was collected as a baseline part of a vaccination study

7 males mean age 33.6 yrs, SD 7.4

1 female aged 30

5.2.2 Samples for analysis

Filtered processed lavage (see thesis appendix) was stored at -70°C prior to analysis. Sera taken on the same day as the WGLF was also stored at -70°C prior to analysis.

5.2.3 Analysis

5.2.3.1 C3

Complement C3 was assayed by competition ELISA as described in chapter 3. Serum and WGLF from the same patient were assayed in the same batch.

5.2.3.2 Other WGLF proteins

IgG, albumin, alpha-1-antitrypsin (A1AT) and haemoglobin (Hb) were assayed as part of a routine battery of tests done on WGLF. Methods are outlined in the thesis appendix. Haemoglobin gave an indication of blood loss, alpha-1-antitrypsin and albumin reflected plasma protein leakage and IgG indicated gastrointestinal inflammation.

5.3 Results

5.3.1 WGLF protein concentrations

TABLE 5.1 Loss of serum proteins to the gut in healthy volunteers

Serum C3 μg/ml	WGLF C3 μg/ml	WGLF IgG μg/ml	WGLF Albumin μg/ml	WGLF A1AT μg/ml	WGLF Hb μg/ml
1,950	0.4	2	9	0	3
1,950	0.3	1	8	0	1
1,500	0.3	1	0	4	2
1,750	0.3	1	1	3	2
1,100	0.3	1	2	3	3
NA	0.3	3	31	19	6
1,750	0.4	NA	17	6	NA
1,200	0.3	NA	11	5	NA

TABLE 5.2 Loss of serum proteins to the gut in patients with inactive Crohn's disease

Crohn's	Serum C3 μg/ml	WGLF C3 μg/ml	WGLF IgG μg/ml	WGLF Albumin μg/ml	WGLF A1AT μg/ml	WGLF Hb μg/ml
4	1,400	0.3	1	4	1	2
1	1,450	2.4	2	10	6	9
1	1,600	0.3	1	1	1	3
1	NA	0.4	1	6	2	2
1	1,850	0.3	1	5	14	38
1	800	3.2	7	8	2	3
2	NA	0.3	4	5	8	3
2	1,100	0.4	1	5	4	2
3	1,450	1.1	1	7	5	6
3	900	0.3	2	7	2	1
3	1,100	0.3	2	6	5	5
3	1,050	0.8	3	5	5	5
3	1,100	1.1	7	15	0	1

Crohn's disease distribution
1 = small bowel
2 = ileo-colonic
3 = colonic
4 = oral

There does not appear to be any trend in WGLF C3 with extent of disease or, from proximal to distal intestine.

TABLE 5.3 Loss of serum proteins to the gut in active Crohn's disease

Crohn's	Serum C3 μg/ml	WGLF C3 μg/ml	WGLF IgG μg/ml	WGLF Albumin μg/ml	WGLF A1AT μg/ml	WGLF Hb μg/ml
1	1,700	9.3	119	27	33	5
1	1,050	2.5	12	49	28	2
1	1,100	21.3	45	122	62	22
2	800	2.8	10	31	5	1
2	1,300	13.3	14	1	24	68
2	1,900	5.6	46	28	11	3
2	1,400	3.8	51	30	14	11
2	1,400	6.8	78	82	21	11
3	1,550	20	72	11	67	87
3	1,300	8.5	34	27	20	1
3	900	2.7	10	6	24	7
3	1,550	5.9	68	20	7	8
3	1,000	3	14	9	10	2

Again no trend in WGLF C3 was seen with the location of CD from proximal to distal. The highest WGLF C3 values occured when WGLF IgG is highest but, there does not appear to be a perfect correlation between the two parameters.

TABLE 5.4 Loss of serum proteins to the gut in patients with inactive UC

UC	Serum C3 μg/ml	WGLF C3 μg/ml	WGLF IgG μg/ml	WGLF Albumin μg/ml	WGLF A1AT μg/ml	WGLF Hb μg/ml
2	650	0.3	6	7	3	3
2	1,100	0.5	2	4	4	7
2	1,250	0.3	1	4	2	5
2	1,100	4.2	5	4	2	22
2	750	0.3	1	2	1	5
2	1,300	0.3	2	4	4	5
1	2,000	0.4	4	6	3	1
1	1,300	0.3	1	6	4	3
1	1,150	1	2	9	4	5
1	1,400	0.5	2	6	6	9
3	1,550	0.5	1	5	3	2
3	1,550	0.3	1	5	1	1
4	1,100	0.3	1	6	7	4

UC disease distribution
1 = left side
2 = pan colitis
3 = proctitis
4 = recto-sigmoid

TABLE 5.5 Loss of serum proteins to the gut in patients with active ulcerative colitis

Disease location	Serum C3 μg/ml	WGLF C3 μg/ml	WGLF IgG μg/ml	WGLF Albumin μg/ml	WGLF A1AT μg/ml	WGLF Hb μg/ml
2	1,200	5.0	24	22	3	2
2	NA	7.8	74	35	42	9
2	1,600	2.5	14	44	13	69
1	1,350	7.0	115	91	18	15
1	1,350	3.5	26	70	8	1
1	1,200	0.5	7	74	12	3
1	1,300	3.0	17	58	9	1
1	1,050	21.0	129	200	45	11
1	800	38.6	107	200	24	12
3	2,050	14.5	131	141	20	14
4	1,300	3.5	16	101	16	31
4	1,450	8.7	27	83	8	1
4	1,000	4.2	8	63	6	4
4	NA	2.9	10	55	5	5

Although the highest WGLF C3 values were seen in the left side UC group (the group with the shortest section of affected colon), the results matched up to the highest WGLF IgG values and so were probably a reflection of the severity of inflammation in a particular patient.

TABLE 5.6 Loss of serum proteins to the gut in patients with non-inflammatory GI diseases

Diagnosis	Serum C3 μg/ml	WGLF C3 μg/ml	WGLF IgG μg/ml	WGLF Albumin μg/ml	WGLF A1AT μg/ml	WGLF Hb μg/ml
1	1,100	0.5	2	4	3	2
1	1,350	1.1	1	4	6	10
1	1,350	0.3	1	1	5	1
1	1,200	0.3	1	2	4	2
1	1,650	0.4	1	1	3	3
1	1,700	0.9	3	6	13	4
1	1,150	0.3	2	2	1	1
1	1,450	0.4	2	2	2	5
1	1,150	0.5	1	5	2	3
1	1,350	0.3	1	5	6	2
2	500	0.3	4	4	1	1
2	NA	0.5	1	3	4	5
3	1,100	0.3	1	1	1	1
3	950	0.5	1	5	5	2
4	1,700	0.3	2	6	3	2
4	1,700	0.3	1	5	7	5
4	1,700	0.3	1	1	3	5
4	1,450	0.3	1	2	4	2
5	1,400	0.4	1	5	1	4
5	1,600	0.3	1	4	8	2
5	1,250	0.3	1	7	6	1
6	NA	0.9	2	23	5	32
6	1,600	0.5	1	5	5	3
6	1,650	0.4	1	5	4	2
6	1,350	1.6	2	13	6	16
7	1,400	0.3	2	5	3	1
7	700	0.4	1	5	3	1
7	1,600	0.3	1	4	1	3

1 = Irritable bowel syndrome
2 = Ischaemic colitis
3 = Haemorrhoids
4 = Other (abnormal liver tests, haemosiderosis, pneumocystic cystoides, systemic lupus erythramatosis)
5 = Constipation
6 = Gastrointestinal bleeding
7 = Functional bowel disorders

Table 5.7 Loss of serum proteins to the gut in patients with non-IBD intestinal disorders with an inflammatory component

Diagnosis	Serum C3 µg/ml	WGLF C3 µg/ml	WGLF IgG µg/ml	WGLF Albumin µg/ml	WGLF A1AT µg/ml	WGLF Hb µg/ml
1	1,050	18.1	2	52	48	9
1	2,000	0.3	1	1	3	2
1	700	0.3	1	1	1	1
1	1,050	2.6	7	28	6	16
1	1,700	0.3	2	8	4	2
1	1,700	0.4	3	13	1	2
1	1,300	0.3	1	3	1	2
1	NA	2.3	9	55	8	4
2	900	0.3	1	6	5	6
2	1,400	0.3	1	1	2	4
2	1,450	0.4	2	9	10	2
3	NA	7.5	23	71	21	2

1 = Cancer/ investigation of cancer
2 = Diverticular disease
3 = Coeliac disease

Three patients with colonic cancer and one with coeliac disease showed raised WGLF C3. Loss of C3 to the gut would therefore appear to reflect intestinal inflammation but not specifically that of IBD.

5.3.2 Statistical analysis

WGLF and serum C3 concentrations were compared between the patient groups. Data was negatively skewed (more cases show low than high values) and so inter-group differences in median C3 values were compared using the non parametric Mann-Whitney test.

5.3.3 'Normal range' for C3 in WGLF

For the purpose of this study, a reference range for WGLF C3 was assigned on the basis of values found in a non-inflammatory disease population as it was hypothesized that individuals with intestinal inflammation would show raised loss of C3 to the gut.

This comprised 28 patients with non-inflammatory intestinal diseases and 8 healthy volunteers. The upper 95th percentile of values from this group gave 0.9 μg/ml as the upper limit of 'normal'.

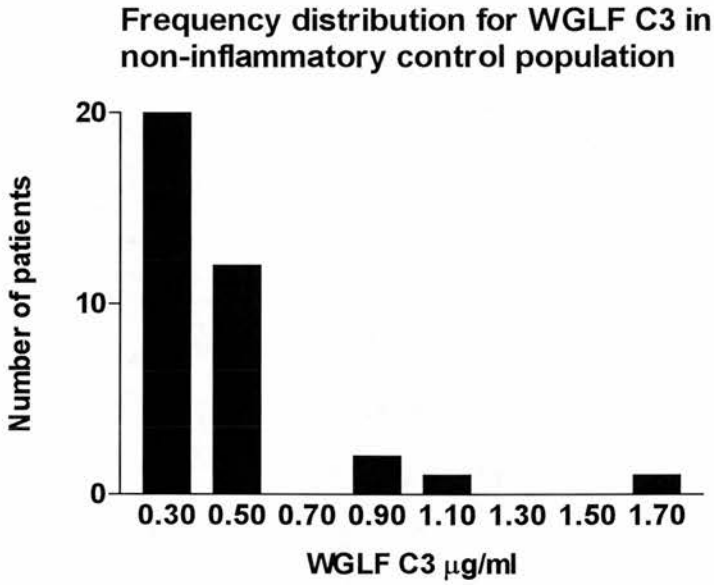

Fig 5.1: Frequency distribution of WGLF C3 values in non-IBD hospital population

5.3.4 Comparison of WGLF C3 in different patient groups

TABLE 5.8 WGLF C3 (median and range) in different patient groups

Patient group	WGLF C3 μg/ml	Number of cases with raised C3	P value (v control)	P value (v other groups)
CDi n = 13	0.4 (0.3- 3.2)	4/13	0.22	v CDa 0.0001
CDa n = 13	5.9 (2.5- 21.3)	13/13	0.0001	v UCa 0.83
UCi n = 13	0.3 (0.3- 4.2)	2/13	0.70	v UCa 0.0001
UCa n = 14	4.6 (0.5- 38.6)	13/14	0.0001	
non-IBD inflammatory disease n = 12	0.4 (0.3-18.1)	4/12	0.42	
non inflammatory Control n = 28	0.3 (0.3- 1.6)			

Most patients with active IBD have raised WGLF C3 whereas fewer patients with inactive IBD or non-IBD intestinal inflammatory conditions do

Compared to the control group of non inflammatory gastro-intestinal diseases and healthy volunteers, patients with active inflammatory bowel disease showed significantly raised loss of complement to the gut. Only one patient with 'active' IBD (WGLF albumin of 74 μg/ml and IgG of 7 μg/ml had WGLF C3 within the reference range. There was some overlap in the inactive IBD groups, two patients with UC and four with Crohn's disease showed raised levels of WGLF complement. This reduced the value of a raised WGLF C3 value to asses whether patients with IBD had active disease.

Predictive values for raised WGLF C3 to predict active disease have not been assigned as the number of cases with inactive disease has been under-represented, increasing the frequency of positive results in the population studied. The presence of raised WGLF C3 in patients with inactive disease could indicate one of three things:

1. C3 could have been an inflammatory parameter that was slow to return to normal once intestinal inflammation has subsided. This is unlikely as raised C3 is likely to exacerbate inflammation.
2. If plasma leakage was the source of C3, cessation of inflammation would bring about closing of the endothelial gaps, with leakage of the larger C3 molecule stopping before that of the smaller albumin. Plasma leakage may not have been the source of WGLF C3 in patients with inactive disease.
3. Raised WGLF C3 in patients with inactive IBD might indicate a pre-clinical change that occured prior to full scale inflammation. This could have potential in predicting whether a patient is likely to have a clinical relapse.

2 and 3 are investigated in later chapters.

5.3.5 Serum C3

Serum C3 values were widely distributed within each patient group but tended to show a rectangular distribution. Differences between the median values were tested with the Mann-Whitney U test.

TABLE 5.9 Serum C3 compared in different patient groups

Patient group	C3 μg/ml	P v control
CDi (13)	1,300 (800-1,850)	0.13
CDa (13)	1,100 (800-1,900)	0.25
UCi (13)	1,250 (650-2,000)	0.10
UCa (12)	1,300 (800-2,050)	0.20
non-IBD inflammatory GI (12)	1,350 (700-2,000)	<0.51
non inflammatory control (36)	1,400 (500-1,950)	

C3 median and range given. Groups compared by Mann-Whitney U test

There was a wide range of C3 values within each patient group, limiting the value of comparing median values. This may have been because of the large intra-assay variability in serum. It was probably due to this that these results do not agree with the findings of Ross et al, 1979 that serum C3 is higher in patients with CD.

5.3.6 Correlation between WGLF IgG and WGLF C3

IgG in WGLF has been shown to correlate well with indices of disease activity in IBD, especially in patients with active disease. It was of interest to see whether WGLF C3 mirrored this relationship. Spearman's rank coefficient was used because WGLF C3 values show a negatively skewed distribution.

TABLE 5.10 Correlation between WGLF IgG and C3

Patient group	WGLF IgG v C3 (r)	P
CDi (13)	0.389	0.038
CDa (13)	0.590	0.275
UCi (13)	0.450	0.094
UCa (14)	0.854	0.005

IgG and C3 are proteins of similar molecular mass (160 and 180 kd respectively). Assuming that gaps between endothelial and epithelial cells were large enough to allow movement of these proteins across the intestinal wall, the quantity of these proteins lost to the gut would be largely dependent on the concentration gradient. This would depend on the serum concentration and the degree of mucosal synthesis. Correlation between concentrations of these two proteins in WGLF would therefore be dependent on the relative importance of these two sources for each proteins. Two factors considered were the relative recovery of the two proteins: evidence from chapter 3 indicates that recovery of C3 may be less than that of IgG. Secondly, the serum concentration of IgG is typically ten times that of C3. There would need to be substantial mucosal synthesis of C3 for WGLF C3 not to mirror IgG.

In inactive IBD there was a weak positive correlation between IgG and C3 in WGLF which just failed to reach significance. Insensitivity at the lower limit of detection of the assays for IgG and C3 may have been a factor. In active ulcerative colitis there is a very strong, highly significant relationship. UC is a disease of the colon and this minimizes differences in recovery of different proteins because the transit time is shorter compared to more proximal protein loss. It is therefore likely that the two proteins were from the same source in patients with active UC. Patients with active CD showed a moderately strong but not significant relationship between WGLF IgG and C3. Two plausible explanations for this could be that this disease shows heterogeneity in terms of the disease process and, also in terms of the site of the disease.

Some patients with more proximal disease might have shown reduced recovery of C3 in WGLF. The positive relationship seen in active IBD has indicated that WGLF C3 reflects disease activity (as measured by WGLF IgG) and so may be a marker of the same inflammatory component.

5.4 Discussion

C3 was detectable in WGLF from most subjects but at levels close to the lower limit of detection in those without intestinal inflammation, as was found with intestinal perfusates from the ileum or jejunum of healthy individuals (Hoj et al, 1981). Loss of C3 to the gut was increased in patients with active Crohn's disease, in agreement of findings with jejunal perfusates (Ahrenstedt et al, 1990). In inactive CD I found WGLF C3 slightly above that of patients with non-inflammatory intestinal conditions but significantly below those of active CD, contrasting with the finding of Ahrenstedt et al that intestinal C3 was similar in both active and inactive CD.
One reason for this may be that their methodology involved perfusion of histologically non-involved regions of the jejunum. It is possible that this avoids those areas with greatest complement loss, whether that be via up-regulated mucosal synthesis or increased vascular permeability with concomitant loss of plasma proteins. Consequently, C3 may have been underestimated in active CD. In contrast, whole gut lavage involves perfusion of the whole gut and will collect complement from both inflamed and non-inflamed regions. This appears to improve the sensitivity of WGLF C3 as a marker of intestinal inflammation.

Their study also demonstrated that C3 was not increased in jejunal perfusates from four patients with ulcerative colitis. The disease activity of these patients was not mentioned and it may have been that these patients had inactive disease.
One flaw in the use of jejunal perfusion to study protein loss in ulcerative colitis is that the disease is primarily a colonic one with little evidence for abnormalities in other areas of the intestine. In my work using whole gut lavage, loss of C3 to the gut was increased in patients with active but not inactive ulcerative colitis.

WGLF C3 was only raised in patients with intestinal inflammation but without being specific for IBD. This study could be improved with larger patient numbers which would allow the impact of extent of disease on loss of C3 to be assessed. WGLF C3 mirrors the results for IgG and so is an objective, if not as specific, marker of disease activity. A more interesting finding was that 4/13 patients with inactive CD had raised WGLF C3 compared with only 2/13 for inactive UC. This may reflect low grade mucosal inflammation during remission.

It is possible that mucosal C3 could be involved in the switch to full grade mucosal inflammation in relapse. The role of raised WGLF C3 in CD as a risk factor for subsequent relapse is assessed in a later chapter.

C3 was quantifiable in serum from all patients using competition ELISA. Median serum C3 concentrations were similar to those observed using ELISA (Ahrenstedt et al, 1990) or radial immunodiffusion (Ross et al, 1979). However, my results give large overlaps between the groups, probably due to poor precision of the competition ELISA in quantifying serum C3.

One reason for this could have been due to C3 in serum being present as intact C3 or converted C3c. As anti C3c was used, this component would be detected to a greater degree. The quantity of C3c detected in serum might depend on conversion invivo or during sample storage. This may explain the failure of my results to confirm previous findings of raised serum C3 in CD.

Other studies of complement in the gut

Other studies of complement in the gut have looked at deposition of complement in the intestinal mucosa. Sections of resected bowel were stained with monoclonal antibodies against C3b or terminal complement complex (TCC). This demonstrated mucous associated C3b and epithelial deposition of TCC in sections from patients with Crohn's disease.

In ulcerative colitis colonic epithelium showed deposition of IgG1 and C3b, possibly indicating an autoimmune reaction with classical complement activation (Halstensen et al, 1992). The intensity of staining for complement was graded subjectively and this showed a weak positive correlation with the histological severity of inflammation.

One problem with this kind of study was that the intestinal inflammation in these patients was probably long-standing and chronic, increasing the likelihood of pathological features representing exacerbated rather than primary insult. It is therefore difficult to make conclusions about the role of complement in intestinal inflammation. Another criticism is that several tissue sections from the same patient were assessed with variation in the grade of inflammation depending on the site.

If complement activation was a feature of inflammation in only some patients, this might add greater weight to the correlation between severity of inflammation and intestinal complement deposition than if only one section per patient was included.

One advantage of WGLF was that endoscopy is performed on suspicion of intestinal inflammation. WGLF complement might reflect early inflammatory changes in relapse. It would be useful to discover whether complement loss to the gut occurs before that of other proteins and to what extent it is produced in the intestinal mucosa. Activation of that complement could reflect a switch between low grade, non pathological inflammation seen in inactive IBD and pathological inflammation with concomitant cellular infiltration and loss of plasma proteins in active disease. The source of WGLF complement will be tested using albumin as a marker of plasma leakage.

CHAPTER SIX

The source of immunoglobulins and complement in the gut

6.1 Introduction

6.1.1 Source of proteins lost to the gut

Detection of plasma proteins in the gut can indicate increased intestinal permeability relating to local inflammation. However, there is evidence that some proteins e.g. IgG and complement can also be synthesized by the gut mucosa. The extent to which WGLF C3 and IgG are locally produced needs to be determined as these proteins have the potential to participate in an inflammatory response.

6.1.2 Albumin as a marker of plasma protein loss to the gut

Albumin has been used as an indicator of plasma leakage as it is not synthesized in the intestine. Although there are other serum proteins which are more stable than albumin e.g. α1AT, there is evidence that this can be produced by enterocytes, making it unreliable as a marker of serum protein loss (Molmenti et al, 1993). Monocytes can also produce α1AT (Van Furth et al, 1983) and these cells are recruited to the intestine in increased numbers in IBD (Burgio et al, 1995). However, other studies show that detection of α1AT in faeces correlates well with excretion of injected radiolabelled albumin (Bernier et al, 1978).

The metabolism of albumin is altered in active IBD with reduced biosynthesis by the liver in chronic disease (Reviewed by Rothschild et al, 1973). With increased loss of albumin to the gut due to changes in vascular and intestinal permeability, serum albumin may be reduced. It was therefore necessary to compare the concentration of WGLF proteins to albumin as a ratio of the serum concentrations.

6.1.3 Aim

It was proposed to compare WGLF concentrations of IgG and C3 to that of albumin, i.e., WGLF IgG /WGLF albumin. Those patients with relatively greater albumin loss probably lose most IgG and C3 from serum. Conversely, patients with relatively greater IgG and C3 relative to albumin might lose a proportion of these proteins from a non-serum source i.e., mucosal biosynthesis. To control for differences in the concentration gradient it was necessary to divide the WGLF concentration by the serum concentration. This gave the relative coefficient of excretion (Jonard et al, 1984):

RCE = [(protein in gut)/(albumin in gut)] / [(protein in serum)/(albumin in serum)] brackets denote the concentration of protein.

By definition the RCE for albumin is 1.0. An RCE greater than 1.0 for the protein of interest indicated that this protein is lost to the gut to a greater degree than was albumin. This may have meant that it was synthesized locally in the intestine or that there is a specific transport mechanism resulting in faster protein loss than could be accounted for by diffusion..

6.1.4 Potential pitfalls in looking at the relative excretion of albumin to other proteins in the gut

A ratio of parameters is subject to error from measurement of any of its components. The use of the ratio value to make conclusions about the nature or source of any of those parameters for a particular population needs to be treated with caution. However, so long as the bias was similar in different patient groups, it may be valid to compare the ratio observed in those groups e.g. to say that relative to albumin, more of protein x was lost to the gut in group 1 compared to 2.
The accuracy of each parameter is dependent on:
1) The specificity of albumin as a marker of plasma loss
2) Recovery and stability of the components
3) Accuracy and precision of measurement of the components

6.1.4.1 Specificity of albumin as a marker of plasma loss

Can albumin loss to the gut be attributed to bleeding ?

Albumin has been used as a marker of plasma leakage, reflecting increased mucosal permeability as a result of inflammation. Increased permeability could have included both mucosal blood vessels and of the epithelial barrier. Intestinal inflammation can also result in direct bleeding into the intestinal lumen via lessions such as ulcers. Proteins lost by this process might not reflect disease activity. To investigate the contribution of bleeding to gastrointestinal plasma leakage a theoretical exercise can show that WGLF albumin was in excess to that attributable to bleeding (as assessed WGLF haemoglobin):

a) Using a WGLF albumin concentration of 30 μg/ml (which was just above the normal reference limit) the extrapolated daily loss of albumin could be calculated: 30 μg/ml x 1000 ml Klean-Prep/h x 24 h = 720,000 μg.

b) From an average serum albumin concentration of 40,000 μg/ml, the quantity of serum lost = 720,000 /40,000 = 18 ml.

c) Assuming an average haematocrit of 45%, this volume of serum represents a blood volume of 100/45 x 18 = 40 ml.

d) From an average blood haemoglobin of 135,000 μg/ml, this volume of blood represents a daily haemoglobin loss of 40 x 135,000 μg/ml = 5,400,000 μg.

e) From the 24,000 ml of Klean-Prep taken per day, this would equate to a WGLF haemoglobin of: 5,400,000 /24,000 = 225 μg/ml.

The % contribution of blood loss to WGLF albumin could be calculated from: (WGLF Hb)/(WGLF albumin) /(225/30) x 100

A plot of % contribution of bleeding to WGLF albumin for cases with raised albumin (>29 μg/ml) shows that in most cases, bleeding only contributes a small fraction.

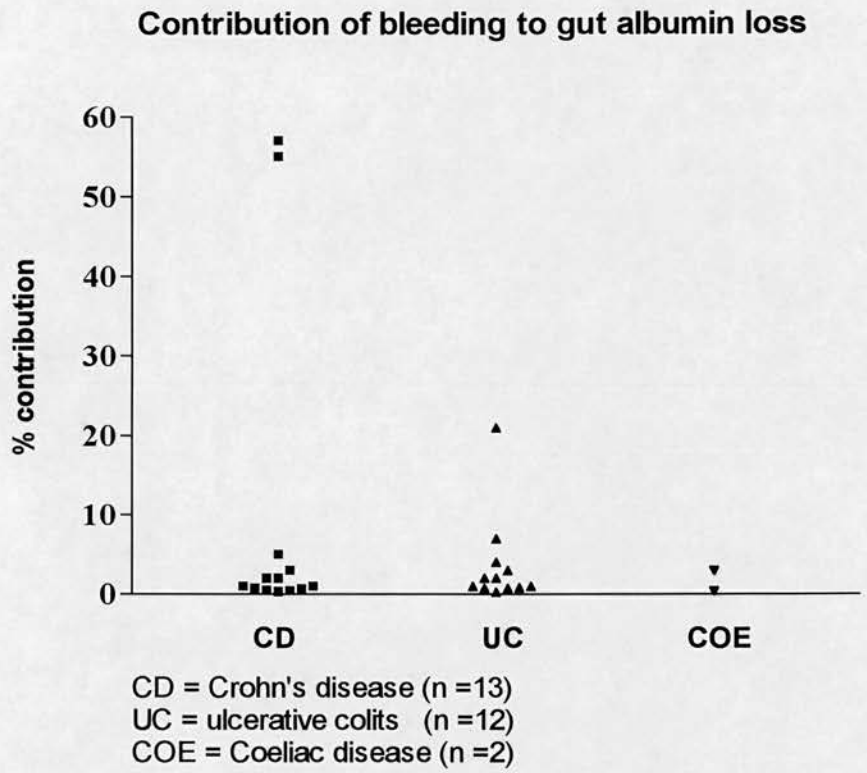

Fig 6.1: Contribution of bleeding to intestinal loss of albumin in intestinal inflammatory conditions

More albumin is lost to the intestine as a result of increased mucosal permeability than is lost due to intestinal bleeding.

6.1.4.2 The stability of proteins in WGLF

As shown in chapter 4, IgG is relatively stable in WGLF. In chapter 5, indirect evidence indicates that C3 is intact. However, Gordon Brydon at the GI unit has shown that albumin is rapidly degraded in WGLF. (Ph.D thesis, University of Edinburgh, 1996). Loss of albumin to the gut might therefore have been underestimated by WGLF albumin.

6.1.4.3 The assay performance of tests to quantify C3, IgG and albumin

a) Measurement of albumin in WGLF

Albumin is quantified in WGLF using an 'in house' immunoturbidity method (described in the thesis appendix). This gives a within batch variation of 8.5 % and between batch variation of 7.4% at 45 μg/ml.

b) Quantification of albumin in serum

Albumin is measured in serum by colorimetric assay, measuring the shift of the reflectance maxima in the dye bromocresol green. The colour complex formed was measured by reflectance spectrophotometer (Vitros, Johnston and Johnston, UK). The department of clinical chemistry at the Western General Hospital, Edinburgh showed a within and between batch CV of 1.4 % for this method.

c) Quantification of C3 in WGLF

Competition ELISA for C3 in WGLF had a between batch CV of 16% and a within batch variation of 6.4%. As this method detected intact as well as converted C3, it is likely to detect all of the C3 present in gut lavage. Recovery experiments indicated that 80% of C3 wasdetected when purified C3 was added to WGLF.

d) Quantification of C3 in serum

C3 was quantified in serum by competition ELISA as described earlier. The between batch variation of the assay is poor with a CV of 31% at 1,400 μg/ml.
This might have introduced errors into the C3 RCE ratio for individual patients. This variability was due to the response curve for serum not being strictly parallel to the C3 standard, giving higher results at greater dilutions. Results for serum C3 were 20% greater than published results (Ahrenstedt et al, 1990).
This was the case for all patient groups studied and so should not bias comparison of the RCE values between the patient groups in this study. Conclusions about C3 secretion based upon the RCE would need to be treated with care because overestimation of serum C3 reduces the apparent RCE, i.e. increasing the component of C3 loss attributable to serum loss.

e) IgG in WGLF

Total IgG in WGLF was assayed by double antibody sandwich ELISA (see thesis appendix) The between batch CV at 7 μg/ml was 8% and the within batch CV was 6%.

f) IgG in serum

IgG in serum was quantified by rate nephelometry using a polymer promoted immune complex reaction. A Beckman Array analyzer was used at the department of clinical biochemistry, Western General Hospital, Edinburgh. The within and between batch CV were less than 4%.

From the performance characteristics of these assays, the RCE for C3 will be most affected by assay variability.

6.1.5 Assumption about the site of protein loss to the gut

It has been assumed that in the IBD patients studied, protein loss to the gut occured from the region of intestine which had been shown to be diseased by radiological and endoscopic examination. Case reports were consulted after the date of the gut lavage to determine changes in the extent of the disease i.e. from colonic to ileo-colonic. In patients with ulcerative colitis, protein loss was assumed to have been from the colon.

6.2 Methods

6.2.1 Influence of WGLF protease activity on albumin recovery

6.2.1.1 Measuring protease activity in WGLF

Azocoll, an insoluble ground collagen which is peptide conjugated to a bright-red azodye has been used to measure protease activity (Chavira et al, 1984). Breaking of the peptide bond by protease activity releases the dye into solution. The colour intensity is measured spectrophotometrically and this is proportional to the protease activity. This method has been used with WGLF to validate the effectiveness of adding protease inhibitors (Gaspari et al, 1988) but it could be adapted to measure the inherent protease activity in unprocessed WGLF. A version of this method is described in the thesis appendix.

Protease activity in WGLF from different disease groups

Patients with IBD might have greater protease activity in their gut lumen due to increased numbers of activated granulocytes. This could influence the recovery of proteins in WGLF from these patients. To assess whether protease activity of WGLF was disease dependent the Azocoll test was performed on unfiltered unprocessed WGLF from the same 49 patients described in this chapter.

These included: 7 patients with inactive Crohn's disease, 6 patients with active Crohn's disease, 7 patients with inactive ulcerative colitis, 6 patients with active ulcerative colitis, 15 patients with non IBD GI disorders with no inflammatory component and 8 patients with non IBD GI diseases with an inflammatory component.

6.2.1.2 Recovery of albumin in WGLF

By quantifying albumin in unprocessed WGLF before and after incubation at 37°C the extent of proteolysis that occured during gut transit can be estimated.

WGLF was collected as samples became available and patient information was not collected.

Procedure

50 ml from the first clear sample of WGLF was collected and stored at 4°C for up to thirty mins before further processing. It was then divided into four aliquots of 10 ml into plastic universal containers. Protein reference standard SPS-01 (PRU, Sheffield, UK) was used as a source of serum albumin (contains 40.3 g/l) for spiking lavage. Aliquots were spiked nil, 6.5, 13 and 26µl of SPS-01 respectively, giving a spiking dose of nil, 25, 50 and 100 µg/ml respectively. Each tube was vortex mixed and the contents divided into 3 ml aliquots.

Instead of using filtration (during which lavage fluid is lost by absorption into filter paper) solids were removed from WGLF by centrifugation at 1,800g for five mins at 15°C. Gordon Brydon's work demonstrated that using centrifugation instead of filtration for the first stage of WGLF processing makes little difference to the residual protease activity of WGLF after processing (Ph.D, University of Edinburgh 1996).

Aliquots from each spiked tube were spun and 2 ml of the supernatant processed as described in the thesis appendix. Processed WGLF was stored at - 70°C in small aliquots. The remaining aliquots of unprocessed spiked lavage were incubated in a waterbath at 37°C for between 30 and 200 minutes before removal and processing. Albumin was assayed in a batch including all samples from a particular recovery experiment, using the immunoturbidity method described in the thesis appendix.

6.2.2 Stability of IgG in WGLF

In an experiment similar to that done with albumin, unprocessed WGLF was incubated for various periods at 37°C before processing.
Either unspiked WGLF or WGLF spiked with IgG from serum (SPS-01 standard) was used. IgG was then assayed and the recovery of IgG calculated relative to WGLF processed at time zero.

6.2.3 Comparison of C3 excretion relative to albumin

Transport of proteins across inflamed endothelium is more dependent on the concentration gradient than the proteins size, contrary to the situation normally (Ballmer et al, 1994). As the serum concentration of albumin is much higher than that of C3, a faster rate of transport of albumin from the blood to the intestine would be expected than for C3. This was tested by calculating the RCE for C3.

6.2.3.1 Patient groups for WGLF C3 analysis

Patients were enrolled retrospectively using a review of the GI database. All WGLF specimens were less than two years old at the time of analysis and had been stored undisturbed at -70°C. Patients with CD were restricted to those with only colonic involvement (as assessed by clinical and radiological examination). This was to try to control for differences in albumin recovery due to the length of intestine that secreted proteins had to travel before collection. This would influence the extent of proteolysis (particularly for albumin).

TABLE 6.1 Patients for study of C3 RCE

6.1a Patients with active colonic CD

	Sex	**Age (yrs)**	**Surgery**	**Prednisalone mg/day**	**Other therapy**
1	M	28	none	30	mesalazine
2	M	45	none	30	codydramol, colifoam
3	M	22	none	no	none
4	M	52	none	20	salazopyrin
5	F	30	none	no	none
6	F	28	none	30	Mesalazine, ferrous sulphate, Azacol

6.1b Patients with inactive colonic CD

	Sex	Age (yrs)	Surgery	Prednisalone mg/day	Other therapy
7	M	30	none	20	none
8	M	34	none	no	none
9	M	72	none	20	Asacol
10	M	51	none	no	none
11	F	61	right helicolectomy	no	Cholestyramine, Azacol
12	F	28	none	9	Mesalazine
13	F	53	None	no	Azocol

patient 12 = patient 6. She was assessed as having active disease on colonoscopy following the first gut lavage (20/05/97). WGLF IgG was 19 μg/ml. On the second colonoscopy (05/06/98) disease was assessed as inactive. WGLF IgG was 1 μg/ml

6.1c Patients with active UC

	Sex	Age (yrs)	Surgery	Prednisalone mg/day	Other therapy
14	M	63	none	no	ferrous sulphate, Sulphasalazine
15	M	55	none	no	Azothioprine, Mesalazine, colifoam
16	M	50	none	30	Mesalazine
17	M	42	none	20	Mesalazine
18	F	40	none	no	Azothioprine, Colifoam, Salazopyrine
19	F	22	ileo-anal pouch	no	none

6.1d Patients with inactive UC

	Sex	Age (yrs)	Surgery	Prednisalone mg/day	Other therapy
20	M	77	none	5	Sulpasalazine, Aspirin
21	M	43	none	no	Mesalazine
22	M	43	none	no	Salazopyine
23	M	32	none	no	Salazopyrine
24	F	75	none	no	Salazopyrine
25	F	71	none	no	Mesalazine, Losec, Bendrofluazide
26	F	44	none	no	Salazopyrine

6.1e Non-IBD gastrointestinal diseases without inflammatory component

Age	Sex	Diagnosis
63	F	HAEMORRHOIDS
62	F	PHEUMOCYSTIC CYSTOIDES
55	F	IBS
54	F	IBS
53	F	IRON DEF ANAEMIA
52	F	IBS
46	F	IBS
38	F	IBS
51	M	ALCOHOLIC
43	M	DIARRHOEA, RECTAL BLEED
39	M	CONSTIPATION
38	M	IBS
34	M	HAEMOSIDEROSIS
29	M	IBS
28	M	DIARRHOEA

6.1f Non-IBD gastrointestinal diseases with inflammatory component

Age	sex	Diagnosis
95	F	FH BOWEL CANCER
75	F	DIVERTICULAR DISEASE
52	F	SLE NSAID INDUCED DIARRHOEA
39	F	ISCHAEMIC COLITIS
37	F	CANCER OF COLON
82	M	REMOVAL OF TUBULAR ADENOMA
72	M	COLONIC DIVERTICULAR DISEASE
61	M	ILEOCAECAL CANCER
49	M	DIVERTICULITIS

6.2.4 Comparison of IgG excretion relative to albumin

The high serum concentration of albumin coupled with its low molecular weight would be expected to result in greater leakage from plasma to the gut than for IgG. However, it is known that there is a large increase in IgG producing plasma cells in the lamina propria of patients with IBD. These might be expected to increase luminal IgG relative to albumin. The same patients as studied for C3 were used.

6.3 Results

6.3.1 Stability of proteins in WGLF

6.3.1.1 WGLF protease activity as measure of albumin degradation

Assay performance

Duplicate tests within the same run gave a CV of 4.6%. Replication of the same sample in a different run gave a CV of 4.9% (Using SD of duplicates = ($\sqrt{\Sigma}$ differences^2 / 2 x number of pairs). The within batch variation was reduced with more replicates, giving a CV of 2.5%.

Protease activity in WGLF from different patient groups

Differences between patient groups were compared by Mann Whitney U test

TABLE 6.2 WGLF protease activity in different patient groups

Disease group	Protease (OD 530 nm)	compare	P value
CDa (6)	0.48 (0.23- 0.69)	v UCa	0.47
CDi (7)	0.54 (0.12-0.59)	v CDa	0.83
UCa (6)	0.57 (0.29- 0.67)		
UCi (8)	0.51 (0.23- 0.78)	v UCa	0.65
non IBD G.I disease without inflammatory component (15)	0.56 (0.08- 0.73)	v CDa	0.45
non IBD G.I disease with inflammatory component (8)	0.58 (0.06-0.64)	v CDa	0.56

Protease activity (median and range)

There was no difference in WGLF protease activity between the patient groups. Despite the numbers of patients being small, the distribution of results was similar in each group. A high WGLF protease result for an individual patient might have resulted in a low albumin because of proteolysis. However, this was unlikely to affect the albumin recovery for the group as a whole and also the relative protein excretion ratio based on the WGLF albumin.

6.3.1.2 Albumin degradation in WGLF

The recovery of albumin in WGLF was calculated in both spiked and unspiked samples for several timepoints. Dilution of WGLF due to processing was taken into account. Graphs of % albumin recovery against incubation time are shown for two WGLF samples. These show the two types of relationship seen, linear or exponential decay type:

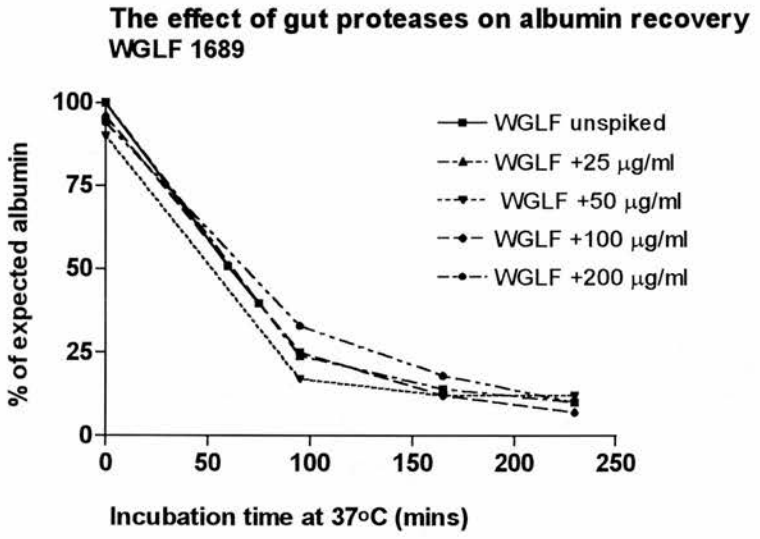

Fig 6.2: Recovery of albumin in unprocessed WGLF incubated at 37°C (a)

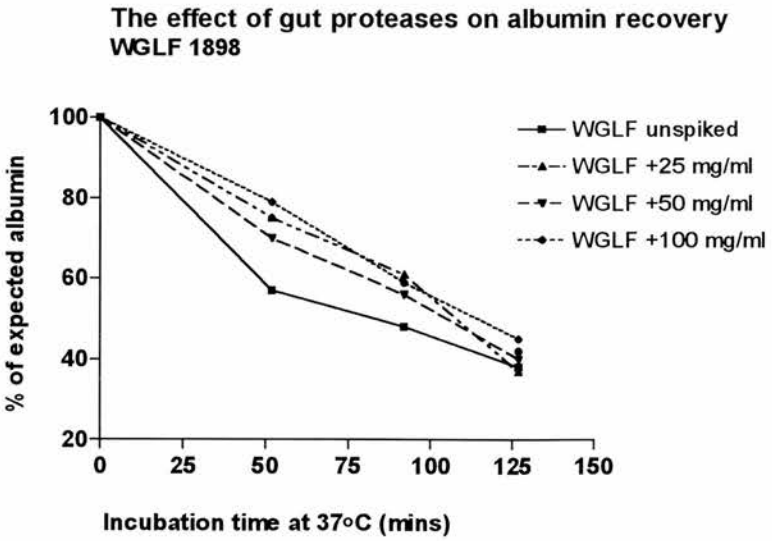

Fig 6.3: Recovery of albumin in unprocessed WGLF incubated at 37°C (b)

In fig 6.2, time zero albumin recovery less than 100 % may indicate very high protease active with proteolysis having occurred during WGLF processing. At longer incubation times the rate of albumin digestion had slown down, possibly indicating enzyme inhibition by digestion products or limited substrate (albumin). Albumin recovery was consistent at different spiking doses but this was not the case with low concentrations of endogenous albumin, possibly reflecting poor assay precision at low concentrations. For each sample, the average % albumin recovery for all the spiking levels was calculated. The rate of albumin digestion by gut proteases varies between individuals.

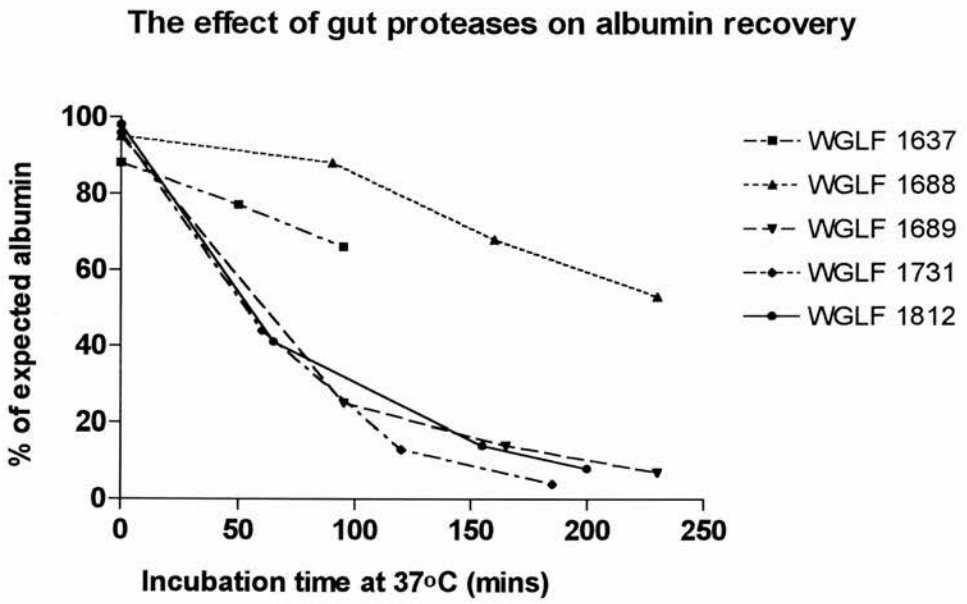

Fig 6.4: Variability in recovery of albumin spiked in unprocessed WGLF and incubated at 37°C. Albumin assayed following WGLF processing (a)

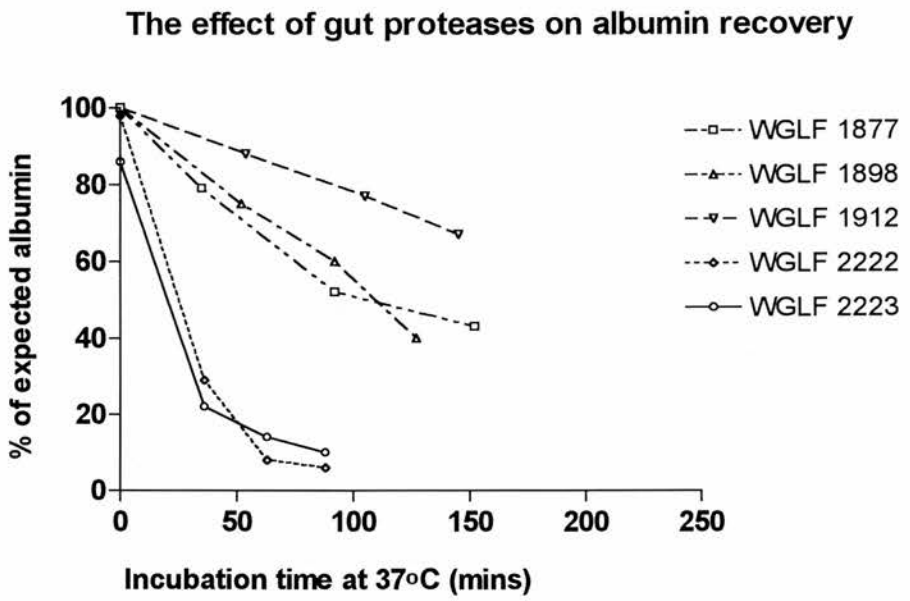

Fig 6.5: Variability in recovery of albumin spiked in unprocessed WGLF and incubated at 37°C. Albumin assayed following WGLF processing (b)

Proteolysis is assumed by the reduction in detection of albumin.

6.3.1.3 Relationship between protease activity and albumin digestion

For comparison of albumin digestion between WGLF samples a reference timepoint of 75 minutes was chosen as this is slightly longer than the time required for colonic transit of WGLF. Average recovery was calculated for each spike at all timepoints and the % albumin recovery plotted against time. For each sample recovery v time data was fitted to an equation using Graphpad Prism (either linear regression or non-linear regression with exponential decay). The percentage albumin recovery after 75 mins was then interpolated from this.

A graph of protease activity of UFUP WGLF against albumin recovery at 75 mins gave a straight line relationship with more albumin being digested (lower albumin recovery) in samples with higher protease activity.

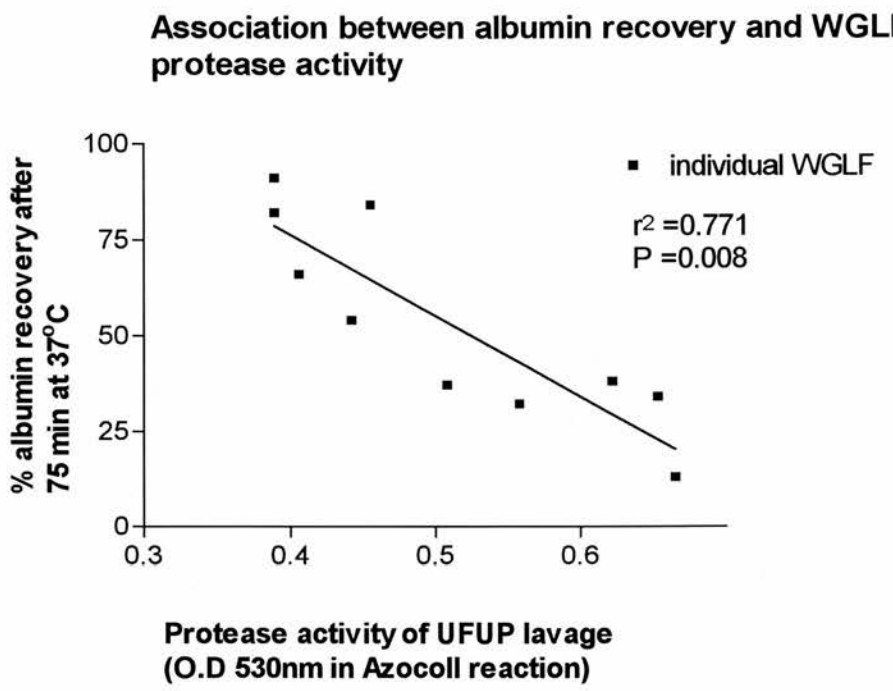

Fig 6.6: Relationship between recovery of albumin in WGLF and WGLF protease activity

Line fitting by linear regression revealed this association to be significant and quite tight, with a coefficient of determination of -0.771. WGLF protease activity therefore gave a good indication as to the likely digestion of albumin during gut transit.

6.3.2 Stability of C3 in WGLF

The stability of C3 in WGLF had not been studied in the same way as albumin because complement fixation might have occured in addition to proteolysis. Also, as a competition assay was used to quantify C3, it was likely that C3 fragments might have given increased C3 recovery. In chapter 3 the plot of C3 detected v protease activity of unfiltered unprocessed WGLF was similar to that for albumin (fig 6.6). This indicated that the quantity of C3 detected in processed WGLF did depend on the protease activity in the gut during transit to a similar degree as did albumin. This relationship was less strong with IgG, suggesting that IgG was more stable in the gut than either albumin or C3.

As very little complement was detected in WGLF from non-IBD patients it was likely that the quantity of complement detected in the gut represented a true finding rather than an artifact due to complement fragmentation..

6.3.3 Stability of IgG in WGLF

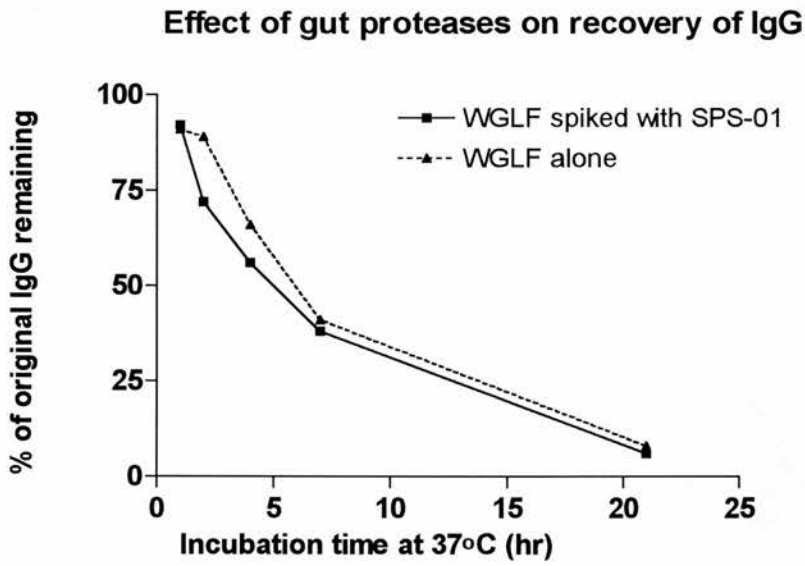

Fig 6.7: Recovery of IgG added to unprocessed WGLF after incubation at 37°C

In a recovery experiment with serum IgG added to unfiltered and unprocessed lavage, 80% of the original IgG was still detected after two hrs incubation at 37°C compared with only 50% for albumin. This confirms indirect evidence that IgG was more stable than albumin.

6.3.4 Influence of protein stability and assay performance on RCE values

From the results of this chapter and that of chapter 3, recovery of WGLF proteins was likely to be greatest for IgG > C3 > albumin. As WGLF albumin is a critical reference point for the RCE calculation, underestimation of this was likely to result in falsely raised RCE values. In calculation of the RCE for C3, this may have been offset by overestimation of C3 in serum (due to lack of parallelism between serum C3 and standard in the competition assay). Care is therefore required in interpreting RCE results from WGLF.

6.3.5 RCE Results

TABLE 6.3 WGLF protein concentrations (median and range)

Patient group	Albumin μg/ml	IgG μg/ml	C3 μg/ml
CDa (6)	83 (6-150)	27.5 (10-161)	12.5 (2.7-30.3)
CDi (8)	7.5 (3-15)	2.5 (1-7)	1.3 (0.3-3.1)
UCa (6)	66.5 (22-200)	25 (8-131)	4.6 (2.5-21)
UCi (8)	5.5 (4-6)	1.5 (1-4)	0.4 (0.3-0.5)
non-IBD GI diseases with no inflammatory component (15)	4 (1-13)	1 (1-2)	0.3 (0.3-1.6)
non-IBD GI diseases with inflammatory component (8)	6 (1-28)	2 (1-7)	0.3 (0.3-2.6)

WGLF IgG was similar in both active CD and UC. It might be inferred that the severity of inflammation was similar in the two groups. Differences in other proteins might therefore have reflected differences in the mechanism of the two disorders. Comparing WGLF C3 in active CD v active CD, no significant difference in WGLF C3 was found ($P < 0.23$, Mann Whitney U test). However when WGLF C3 was compared between inactive CD and inactive UC a significant difference was seen in ($P < 0.0074$). This might implicate complement in the early inflammatory reaction in Crohn's disease but not in UC.

TABLE 6.4 CDa WGLF proteins

	Serum IgG µg/ml	Serum Albumin µg/ml	Serum C3 µg/ml	WGLF IgG µg/ml	WGLF Albumin µg/ml	WGLF C3 µg/ml	IgG RCE	C3 RCE
1	6,300	35,000	1,450	12	87	6.7	0.8	1.9
2	11,100	37,000	1,750	36	79	18.2	1.5	4.9
3	15,200	31,000	1,800	161	111	30.3	3	4.7
4	12,100	37,000	900	10	6	2.7	5	18.5
5	16,300	37,000	2,150	56	150	27.2	0.8	3.1
6	10,400	42,000	3,150	19	29	6.1	2.6	2.8

TABLE 6.5 CDi WGLF proteins

	Serum IgG µg/ml	Serum Albumin µg/ml	Serum C3 µg/ml	WGLF IgG µg/ml	WGLF Albumin µg/ml	WGLF C3 µg/ml	IgG RCE	C3 RCE
7	10,000	47,000	1,950	2	9	1.7	1	4.6
8	12,600	39,000	1,100	2	6	0.3	1	1.8
9	9,960	38,000	1,050	3	5	0.8	2.3	5.7
10	17,500	42,000	1,100	7	15	1.1	1.1	2.8
11	12,600	44,000	2,700	5	11	3.1	1.6	4.6
12	7,810	48,000	2,550	1	9	1.4	0.7	2.9
13	14,400	44,000	2,100	1	4	1.7	0.8	8.9

TABLE 6.6 UCa WGLF proteins

	Serum IgG µg/ml	Serum Albumin µg/ml	Serum C3 µg/ml	WGLF IgG µg/ml	WGLF Albumin µg/ml	WGLF C3 µg/ml	IgG RCE	C3 RCE
14	8,740	40,000	1,600	14	44	2.5	1.5	1.4
15	16,900	31,000	1,050	129	200	21.0	1.2	3.1
16	15,300	42,000	1,000	8	63	4.2	0.3	2.8
17	10,300	42,000	2,050	131	141	14.5	3.8	2.1
18	13,800	40,000	1,350	26	70	3.5	1.1	1.5
19	17,900	38,000	1,200	24	22	5.0	2.3	7.2

TABLE 6.7 UCi WGLF proteins

	Serum IgG µg/ml	Serum Albumin µg/ml	serum C3 µg/ml	WGLF IgG µg/ml	WGLF Albumin µg/ml	WGLF C3 µg/ml	IgG RCE	C3 RCE
20	13,700	41,000	1,250	1	4	0.3	0.7	2.5
21	9,950	41,000	1,100	1	6	0.3	0.7	1.9
22	8,780	46,000	1,400	2	6	0.5	1.7	2.7
23	15,400	41,000	1,300	2	4	0.3	1.3	2.4
24	13,980	39,000	2,000	4	6	0.4	1.9	1.3
25	14,100	45,000	1,300	1	6	0.3	0.5	1.7
26	12,700	43,000	1,100	2	4	0.5	1.7	4.9

TABLE 6.8 Serum protein concentrations

Patient group	Albumin mg/ml	IgG mg/ml	C3 mg/ml
CDa (6)	3.7 (3.1-4.2)	11.6 (6.3-16.3)	1.8 (0.9-3.1)
CDi (8)	4.3 (3.8-4.8)	12.6 (7.8-17.5)	1.9 (1.1-2.7)
UCa (6)	4.0 (3.1-4.2)	14.6(8.4-17.9)	1.3 (1.0-2.1)
UCi (8)	4.2 (3,9-4.6)	13.2 (8.0-15.4)	1.3 (1.1-2.0)

No significant difference was seen in serum C3 between patients with active CD and those with active UC ($P = 0.23$), contrasting with the finding by others of raised serum C3 in CD as a result of the acute phase response (Ross et al, 1979). The discrepancy was probably due to the wide spread of my data and the poor precision of the assay for C3 in serum. The finding of slightly reduced serum albumin in patients with active CD as opposed to inactive CD ($P = 0.01$) could reflect reduced liver synthesis combined with loss of albumin to the gut.

6.3.5.1 Relative loss of C3 compared to albumin

TABLE 6.9 RCE for C3 relative to albumin

Patient groups	RCE for C3	compare	P value
CDi (8)	4.6 (1.8-9)	CDi v CDa	0.81
CDa (6)	3.1 (1.9-4.9) *	CDa v UCa	0.42
UCi(8)	2.4 (1.3-4.9)	UCi v CDi	0.06
UCa (6)	2.5 (1.4-7.2)	UCa v UCi	0.72
All CD (14)	3.8 (1.8-8.9)	All CD v All UC	0.04
All UC (14)	2.4 (1.3-7.2)		

* One outlier with RCE of 18.5 omitted

The RCE values were much higher than the 1.0 predicted to represent protein loss as a result of plasma leakage alone. It was likely that underestimation of WGLF albumin because of proteolysis during gut transit was the major factor in this.
If 50% of albumin in the gut was degraded, the apparent RCE would be twice the expected i.e. a ratio of two may point to serum being the major source of that protein.

The RCE values obtained for C3 loss in patients with UC patients were just above two, possibly indicating loss from serum. Patients with CD show a higher excretion ratio which may indicate that some of the C3 lost to the gut was produced in the intestinal mucosa. The RCE was greatest in inactive CD, prompting the suggestion that mucosal synthesis of C3 represents a fundamental metabolic change in CD and not solely a feature of active inflammation.
In this group of CD patients, serum C3 was raised and serum albumin depressed when compared with UC. These would tend to lower the RCE value in CD, making it unlikely that the finding of raised RCE for C3 in CD was an artifact. The finding that the RCE was slightly reduced in active CD as compared with inactive might be explained by increased plasma leakage in active CD. This data supports findings from segmental perfusion studies of mucosal complement production in Crohn's disease (Ahrenstedt et al, 1990).

Patients with inactive CD secrete pro-inflammatory cytokines to the gut, providing evidence for a persistent low grade inflammatory process within the mucosa (Arnott et al, 1997). It is possible that once a threshold level for production of inflammatory mediators such as cytokines and complement is reached, full blown intestinal inflammation ensues. In support of this hypothesis, those patients with inactive CD who had greater quantities of cytokines such as IL-1 in their WGLF had an increased risk of clinical relapse (Arnott et al, 1998).

In a subsequent chapter, complement in WGLF as a potential predictor of clinical relapse was considered. A possible mechanism of relapse based on macrophage production of IL-1 with subsequent synthesis of C3 is discussed. Activation complement may produce chemotactic fragments such as C3a which could attract neutrophils to the gut with inflammatory consequences.

6.3.5.2 Intestinal loss of IgG relative to albumin in patients with IBD

Results are given as median and range and differences between the groups tested by the Mann Whitney U test.

TABLE 6.10 Comparison of RCE for IgG in different patient groups

	IgG RCE	Compare	P value
CDi (7)	1.1 (0.7-2.6)	v CDa	0.52
CDa (6)	1.5 (0.8-3) *	v UCa	0.57
UCi (7)	1.2 (0.5-1.9)	v CDi	0.79
UCa (6)	1.3 (0.3-3.8)	v UCi	0.65

* One outlier with an RCE of 5 has been removed. This same patient had an RCE for C3 of 18.5

The RCE for IgG assessed using WGLF was comparable to the ratio of 1.17 obtained from colonic perfusion of healthy subjects (Prigent-Delecourt et al., 1995) in whom most IgG in the gut would have been expected to be plasma derived because of the relatively paucity of IgG B-cells.

Patients with IBD have increased IgG plasma cells in the intestinal lamina propria (Rosekrans et al, 1980) which might contribute to IgG lost to the gut i.e. a raised RCE. These B-cells produce increased amounts of IgG in active IBD.

The finding that RCE for IgG was not increased in active disease was therefore unexpected.

In a previous study using colonic washings a similar comparison between IgG and albumin in the gut was made and it was concluded that leakage of serum contributed a relatively low % of IgG found in the washings (Macpherson et al, 1996). This might be explained by their use of the equation:
{(albumin (washings) /(albumin (serum)}/[(IgG (washings)/(IgG (serum)]
If the fraction resulting from this was taken to be the fraction of gut IgG derived from serum this is incorrect as the lower term (IgG (washings)/(IgG (serum) actually gave the proportion of IgG which is mucosally derived.

As it is undisputed that mucosal B-cells produce more IgG in active IBD, it is difficult to explain these results. One explanation could be that the RCE is a relatively insensitive indicator of mucosal protein synthesis when the serum concentration of that protein is relatively high. A change in the RCE for IgG from 1 to 2 would require ten times the mucosal synthesis of IgG that would be required with C3 to produce a similar shift in the RCE value. There may therefore be significant mucosal synthesis of IgG in active IBD. It would be of interest to know whether production of IgG is an early event in relapse with the possibility that mucosal IgG contributes to this. IgG in the gut mucosa is also likely to exacerbate intestinal inflammation via interaction with complement and Fc receptors of immune cells.

Another explanation for the failure to show a raised IgG RCE in active disease could be a type 2 statistical error resulting from the number of patients studied being too small.

6.3.5.3 The effect of steroids on the RCE value

Steroids down-regulate mucosal immune responses and this might have influenced the apparent mucosal contribution to WGLF C3 and IgG, Patients with CD who were on steroids were compared with those who were not.

TABLE 6.11 Comparison of RCE for C3 and IgG in CD patients on steroids v CD patients not on steroids

	Active: Inactive	**RCE C3**	**P value**	**RCE IgG**	**P value**
CD steroids	4:3	4.6 (1.9-18.5)	0.73	1.5 (0.7-5)	0.78
CD non	2:4	3.9 (1.8-8.9)		1.1 (0.8-3)	

These results show that the source of IgG or C3 lost to the gut in patients with CD is unlikely to be affected by whether they were taking steroids (prednisolone) or not.

6.3.5.4 Day to day variation in the pattern of protein secretion

WGLF protein measurements represent a single 'snapshot' of secretion in the intestine. It is possible that day to day variation in protein loss to the gut might account for small differences seen in the RCE between some disease groups. RCE calculations for IgG based on WGLF results from more than one day were compared.

TABLE 6.12 Inter-day variation in RCE values for IgG

Patient	Day	IgG RCE	WGLF IgG μg/ml
A	1	5.75	14
A	2	5.53	6
B	1	3.81	16
B	2	1.88	8
C	1	2.63	9
C	2	2.63	11
D	1	2.65	19
D	2	2.93	4
E	1	1.37	56
E	2	1.32	61
E	3	1.35	56
F	1	2.52	8
F	2	2.32	24
G	1	1.27	2
G	2	1.86	4
H	1	1.19	37
H	2	0.84	6

Comparing the RCE on day one and two gives:

Day 1 n =8 median RCE = 2.6

Day 2 n =8 median RCE = 2.1 P = 0.75 (Mann-Whitney U test)

It is therefore unlikely that day to day variation in protein loss had a significant effect on the RCE for a particular patient. From this, comparison between patient groups for the RCE taken from a single WGLF appears to be valid.

6.4 Discussion

This study has shown that it is difficult but nevertheless possible to form conclusions about the source of proteins lost to the gut when using albumin as a marker of plasma leakage. Although the actual RCE values may be inaccurate because of factors affecting recovery or quantification of proteins, it may be valid to compare values in different patient groups.

Patients with active IBD appeared to lose more IgG to the gut through plasma leakage than from mucosal synthesis of IgG. This was an unexpected finding since there are a greatly increased number of IgG producing B-cells in the intestinal mucosa of patients with active IBD. Without having a non biological marker (e.g. radioisotope) for IgG from serum, it was difficult to identify IgG lost to the gut as serum or mucosal derived.

The intestinal mucosa of patients with IBD contained increased numbers of IgG B cells, as demonstrated by immunohistochemical study of the colon (Baklien and Brandtzaeg, 1975). Immunohistochemical studies of the IgG subclasses in the intestine of patients with inactive IBD demonstrated that IgG1 B cells were increased in proportion to their fraction in blood (Iizuka, 1990). These cells might have been recruited to the intestine during inflammation. They could produce more IgG during active disease in response to pro-inflammatory cytokines. IgG has the capacity to exacerbate intestinal inflammation via interaction with Fc receptors and complement.

Loss of complement to the gut has shown a predictable finding of increased loss in active IBD, reflecting increased loss of plasma proteins. However, the finding of raised WGLF C3 in patients with inactive CD but not UC presented an interesting finding of a difference in the early inflammatory reaction of the two diseases. Some of this C3 was likely to have been synthesized within the intestinal wall as the RCE for C3 was raised in patients with inactive CD, supporting previous findings (Ahrenstedt et al, 1990).

This might support an interesting hypothesis that remission in CD represents a state of low grade inflammation and that relapse might represent breach of a threshold level for intestinal production of complement and cytokines. A longitudinal study of WGLF C3 in individual patients might yield interesting results, especially if an increase was seen prior to clinical relapse.

The role of C3 in intestinal inflammation is yet to be elucidated. However, the finding of increased intestinal C3 without concomitant increased IgG might suggest that if complement activation was important in relapse of CD, it might not be dependent on IgG i.e. the alternative pathway.

Evidence for this came from the finding that deposition of C3b on the intestinal epithelium was not associated with IgG in Crohn's disease (Halstensen et al, 1992).

These results did not allow in depth speculation on the role of complement in ulcerative colitis. However, as may be the case with CD, increased loss of complement to the gut in active disease may perpetuate intestinal inflammation through complement activation. Evidence for intestinal deposition of complement in ulcerative colitis has shown that C3b was associated with IgG. This may indicate that the classical pathway of complement activation is involved (Hakstensen et al, 1992).

CHAPTER SEVEN

WGLF C3 as a predictor of relapse in Crohn's disease

7.1 Introduction

Crohn's disease is characterized by periods of clinical remission, when symptoms may be modest and intestinal inflammation resolves, interspersed with clinical relapse with worsening symptoms and mucosal inflammation. Therapy in Crohn's disease seeks to prolong the periods of remission and decrease the periods in relapse. Where inflammation becomes very severe, surgical intervention may be necessary. There are inherent risks associated with surgery e.g. infection. The risk of future relapse is also increased following surgery. Earlier detection of relapse might avert the need for surgery.

Clinical indices based on a physicians assessment of the patient e.g., the Crohn's disease activity index (Best et al, 1976) are not sensitive enough to detect early signs of relapse. Since this is a measure of patient well-being judged on subjective factors and the number of stools per day it can give a high result even if there is no evidence of intestinal inflammation. e.g., in patients with intestinal obstruction or psychological problems. Objective markers of intestinal inflammation e.g. WGLF IgG (Choudari et al, 1993) have been used to assess disease activity but, by the time significant changes are seen, the patient may have already relapsed. A more sensitive indicator is required.

Previous studies have examined serum proteins of the acute phase response in Crohn's disease. There is a rapid change in the serum protein, C-reactive protein (CRP) and this might be a useful indicator of relapse. One study has shown that patients with inactive Crohn's disease who had not relapsed over a one year period were more likely to relapse in the second year if their CRP remained high over that time (Boirivant et al, 1988) .

This study lacked sensitivity as one third of patients in remission and one third of patients with active disease had elevated CRP. Raised CRP can also be increased during microbial infections and is not specific for relapse of Crohn's disease. It may also be more relevant to look for a marker of relapse released from the intestine i.e. is a direct indicator of intestinal inflammation.

The value of a biological marker in predicting clinical relapse reflects its early release. In one study, intestinal tissue was taken from surgical resections and found to contain high concentrations of IgG (Heimann, 1983) pinpointing high tissue IgG as a predictor of relapse. However, an earlier index prior to requirement for surgery would facilitate less radical therapy.

7.1.1 Whole gut lavage proteins

IgG

IgG in WGLF has been established as an objective measure of disease activity by prospective comparison with the CDAI, showing good correlation with the CDAI in active disease i.e. CDAI >150. The cut off value for WGLF IgG of 10 μg/ml was established from a cohort of patients with non-inflammatory bowel disorders. Conveniently, this cutoff value corresponded to a CDAI of 150 (Choudari et al, 1993). Of 67 patients with CD(30 active), 8 had WGLF IgG of between 5 and 9 μg/ml and only one of these showing signs of active disease by a raised WGLF albumin. This indicated that WGLF IgG was relatively insensitive for detecting minor inflammatory changes that may be a prelude to relapse.

7.1.2 Inflammatory cytokines

IL-1β and IL-8

The transient and local nature of pro-inflammatory cytokines made them ideal candidates to study as indicators of tissue inflammation. Measuring WGLF concentrations might reveal early inflammatory events in the intestine.

IL-1β is produced by macrophages which have taken up foreign material. It is a pivotal cytokine in directing the immune response, promoting T-cell proliferation via up-regulation of IL-2 receptors. It also up-regulates expression of adhesion molecules such as ICAM-1 on vascular endothelium (Wyble et al, 1996) facilitating recruitment of immune cells from the circulation to the site of inflammation.

IL-8 is produced by many cell types, including intestinal epithelial cells. It recruits neutrophils to the site of inflammation as a first line back-up to clear foreign material by phagocytosis.

Most patients with active CD had high concentrations of IL-1β and IL-8 in their WGLF, reflecting active inflammation. Some patients with inactive CD also had raised cytokine concentrations in WGLF, possibly indicating low-grade inflammation or the onset of relapse. It was postulated that it was these patients who were at greater risk of relapse.

To test this hypothesis, patients with clinically assessed inactive CD underwent the gut lavage procedure. IgG, IL-1β and IL-8 were assayed in the WGLF by ELISA. Patients were then followed-up over a one year period and assessed as possible relapse (change in symptoms requiring steroid therapy) or as in remission. Patients were stratified according to the concentration of WGLF proteins at initial investigation and the number of patients relapsing compared in the high and low WGLF protein groups.

Those patients with raised pro-inflammatory cytokines in WGLF were at a significantly increased risk of relapse during the follow-up period (Arnott et al, 1998).

7.1.3 Increased mucosal permeability and relapse in Crohn's disease

Dr Ian Arnott's work at the unit assessed intestinal permeability in patients with inactive CD (CDAI <150) by urine excretion of oral administration of lactulose and rhamnose. A significantly greater proportion of patients had raised intestinal permeability compared with controls i.e. raised intestinal permeability was a feature of Crohn's disease (MD thesis, University of Manchester, 1998). The change in sugar ratio was mainly because of increased permeability to the large lactulose probe, reflecting increased access to the crypts. This is known to be the main route for uptake of macromolecules.

Increased mucosal permeability might be a logical reason for relapse in CD because of the increased access of luminal antigen to the mucosal immune system. Foreign material is taken up by macrophages which are then activated to produce IL-1β and possibly C3. Cytokines such as IL-1β are known to increase vascular permeability and might further increase intestinal permeability (Burke-Gaffney and Keenan, 1993). Antigens are presented to T-cells, some of which might be activated to orchestrate an immune response. In the environment of the intestinal mucosa, this is easily amplified with resultant inflammatory damage to the tissue.

7.1.4 C3 as a potential predictor of relapse in Crohn's disease

In a previous chapter it was shown that some patients with inactive CD (assessed by WGLF IgG) had raised WGLF concentrations of C3. In these patients, WGLF albumin was generally low, possibly indicating that plasma leakage did not account for all of the WGLF C3 seen.

It is known that cells within the intestinal mucosa can synthesize C3 e.g., macrophages (Hetland et al, 1999), (Beatty et al, 1981) or epithelial cells (Moon et al, 1997), with this being augmented by pro-inflammatory cytokines such as IL-1β. Consequently, it might be expected that WGLF C3 would mirror WGLF IL-1β in predicting relapse of CD. A mechanism of inflammation involving activation of mucosal complement might have been a factor in relapse of CD.

7.1.5 Aims

The aim of this study was to determine whether WGLF C3 was useful in identifying those patients with CD in remission who were at high risk of future relapse; and also to confirm that raised WGLF IL-1β predicted relapse.
The relationship between the role of WGLF IL-1β and C3 to clinical relapse of CD was examined. This has been used to show whether increased mucosal C3 is linked to raised IL-1β, providing further information about the inflammatory process in CD.

7.2 Methods

7.2.1 Whole gut lavage

Whole gut lavage was performed as described in the thesis appendix, in most cases as a bowel preparation prior to investigation by colonoscopy. In selecting the number of patients to follow up, previous prospective studies of Crohn's disease were considered. Dr Ian Arnott had found that one third of Crohn's disease patients relapsed within one year of gut lavage. We considered fifteen cases of relapse as sufficient to perform chi square statistics on whether relapse was related to high or low WGLF C3. Forty five patients were therefore considered adequate for the study.

7.2.2 Patient follow-up

Patients were followed up until clinical relapse or up to one year post gut lavage (Dr I Arnott, WGH, Edinburgh).
The patient status was designated as follows:
1) Remission. Not on steroids or immunosuppressives for at least 6 months prior to assessment.
2) Resection. Major surgical intervention with bowel resection.
3) Immunosuppressives. Either continuation or requirement for azothioprine or cyclosporin.
4) Relapsing. At least one clear relapse needing the introduction of oral steroid therapy.

5) Steroid dependent. On oral steroid therapy for nine out of 12 months following WGL.

7.2.3 WGLF assays

Interleukin-1β (IL-1β)

IL-Iβ was assayed using a commercially available sandwich ELISA kit (Cistron Biotechnology, New Jersey, USA). This was performed by Mrs H.Drummond/ Dr I.Arnott as detailed in the chapter appendix.
I have investigated the relationship between WGLF IL-1β and relapse in my cohort of patients to support previous work by Dr Ian Arnott using a separate cohort at an earlier date (Arnott et al, 1998). This was to compare follow-up between two groups of patients with CD and so validate my cohort for consideration of monitoring disease outcome in relation to WGLF C3.

C3

C3 in WGLF was assayed using the competition ELISA described previously.

Blinding

A blinded protocol of sample identification using identifier numbers rather than patient name was adopted to prevent bias and analysis performed after follow-up to prevent clinical assessment bias through knowledge of laboratory parameters.

7.2.4 Patients

There were 42 patients with inactive CD (as assessed by CDAI or were not appropriate, by WGLF IgG). There was a discrepency with the 43 patients quoted in the study abstract (British Society of Gastroenterology, Birmingham, 2000). This was an error and it should have been 42 patients with one patient having been studied on two separate occaisions. This 33 year old man with ileocolonic disease required immunosuppressents one year after follow up (WGL 2/8/96).

However, if the follow-up period had been taken after a second WGL (2/11/96) he had relapsed within the year. As the numbers for patients relapsing were small, I have decided to include only the data from this second follow up-period.

A total of 42 patients with inactive CD (assessed by WGLF IgG <10 μg/ml)
22 females, mean age 46.7, SD 14.1 yrs
20 males, mean age 52.3, SD 16.3 yrs

TABLE 7.1 Patients with inactive Crohn's disease who were clinically followed-up for one year after undergoing whole gut lavage

Sex	Age	Disease duration months	Resections	Disease outcome	Pred mg/day	Azo	5ASA	Steroid enema
F	32	2	0	dependent	0	N	N	N
F	50	152	0	dependent	5	Y	N	N
F	63	316	2	dependent	2.5	Y	N	N
F	65	44	0	dependent	7.5	N	Y	N
M	60	31	0	dependent	5	N	N	N
M	63	366	1	dependent	10	Y	N	N
M	69	5	0	dependent	10	N	N	N
M	20	27	0	immunosup	0	Y	N	N
M	34	New	0	immunosup	0	Y	N	N
F	28	23	3	lost	0	N	N	N
F	52	New	2	lost	0	N	N	N
M	56	95	0	lost	0	N	N	N
M	80	New	0	lost	5	Y	N	N
F	20	3	0	relapse	0	N	Y	N
F	31	56	1	relapse	0	N	N	N
F	34	143	2	relapse	0	N	Y	N
F	57	321	3	relapse	10	N	Y	N
F	57	310	3	relapse	0	N	N	Y
M	33	32	0	relapse	30	Y	Y	N
M	34	72	1	relapse	0	N	N	N
M	36	4	0	relapse	0	N	N	N
M	37	1	0	relapse	40	N	N	Y
M	54	250	2	relapse	0	N	Y	N
F	33	103	0	remission	0	N	Y	N
F	43	180	0	remission	0	N	Y	N
F	54	234	0	remission	0	N	Y	N
F	54	359	2	remission	0	N	Y	N
F	54	62	1	remission	0	N	N	N
F	55	316	4	remission	0	N	N	N
F	59	298	2	remission	0	N	N	N
F	59	338	1	remission	0	N	Y	Y
M	53	185	3	remission	0	N	Y	N
M	58	New	0	remission	0	N	Y	N
M	62	374	3	remission	0	N	N	N
M	72	22	0	remission	0	N	N	N
M	76	14	0	remission	0	N	Y	N
F	29	58	2	resection	5	N	Y	N
F	33	118	2	resection	0	N	N	N
F	67	218	3	resection	5	N	Y	N
M	42	128	2	resection	15	N	N	Y
M	51	77	1	resection	0	N	N	N
M	57	74	3	resection	0	N	N	N

Pred = prednisolone, Azo = Azothioprine, Immunosup = immunosuppresent, Y = on drug, N = not on drug

This table shows that those patients requiring immunosuppressive therapy at the start of the follow-up period were more likely to relapse. Some patients were new cases.

7.2.4.1 Clinical diagnosis of Crohn's disease

There are four features used to assign a diagnosis of CD:

1) A clinical history of abdominal pain, diarrhoea, nausea or loss of body weight

2) Mucosal abnormalities seen radiologically e.g. narrowing of gut lumen or endoscopically e.g. apthous ulcers, cobblestone mucosa and strictures.

3) Occurrence of fistulae or abscesses.

4) Histological findings including transmural lymphocytic infiltration and the presence of epithelial granulomas.

At least 2/4 features must be present for a diagnosis of Crohn's disease to be made. Several clinicians at the Western General Hospital have been responsible for making the diagnosis for patients included in this study.

7.2.5 Data analysis

Patients were subdivided on the basis of WGLF C3 or IL-1β. Differences in the frequency of patients who relapsed were examined by chi square test and by Kaplan-Meier survival analysis (Graphpad Prism software) by log rank test. Associations between WGLF parameters were assessed by correlation (Pearson).

7.3 Results

TABLE 7.2 Patients who relapsed

Age	Sex	WGLF C3 μg/ml	WGLF IL-1β pg/ml	Days to relapse	CDAI
20	F	0.6	7	UN	86
31	F	0.3	66	111	132
34	F	2.2	16	249	92
57	F	0.5	21	184	*
57	F	0.4	10	187	*
33	M	0.7	535	81	60
34	M	0.8	38	95	137
36	M	0.8	148	14	103
37	M	0.3	18	101	81
54	M	3.7	37	202	62

UN = patient relapsed but the date at which this occurred was not accurately noted. This patient was therefore excluded from Kaplan-Meier survival curve analysis.
The CDAI was not assessed in two patients who had subtotal colectomy as bowel function (number of loose stools) is not meaningful.

TABLE 7.3 Patients remaining in remission

Age	Sex	WGLF C3 μg/ml	WGLF IL-1β pg/ml	CDAI
33	F	1	6	86
43	F	1.5	10	85
54	F	2.1	4	75
54	F	0.9	16	124
54	F	1.4	49	136
55	F	0.4	19	NA
59	F	1.3	2	25
59	F	1.4	72	51
53	M	1.1	10	79
58	M	0.6	14	123
62	M	0.7	16	NA
72	M	0.8	18	40
76	M	0.3	9	91

In two cases (NA) the CDAI was not calculated. The first had an ileostomy and the second had colectomy with major ileal resections.

TABLE 7.4 Patients requiring surgery

Age	Sex	WGLF C3 μg/ml	WGLF IL-1β pg/ml	CDAI
29	F	2.1	32	NA
33	F	2.7	36	50
67	F	0.8	19	NA
42	M	0.5	6	65
51	M	1.1	31	NA
57	M	0.3	7	NA

NA = these patients either had coloctomy or ileostomy prior to the study. The CDAI is not an accurate measure of disease activity in these patients

TABLE 7.5 Patients remaining steroid dependent

Age	Sex	WGLF C3 μg/ml	WGLF IL-1β pg/ml	CDAI
32	F	1	13	133
50	F	1.1	26	149
63	F	1.1	12	134
65	F	1.8	13	102
60	M	0.3	7	81
63	M	0.4	12	41
69	M	3.2	10	102

TABLE 7.6 Patients requiring immunosuppressants

Age	Sex	WGLF C3 μg/ml	WGLF IL-1β pg/ml	CDAI
20	M	1.4	104	79
34	M	0.3	12	44

TABLE 7.7 Patients lost to follow-up

Age	Sex	WGLF C3 μg/ml	WGLF IL-1β pg/ml	CDAI
28	F	2.7	144	145
52	F	0.5	NA	ND
56	M	0.5	29	111
80*	M	0.3	6	ND

* Patient died shortly after WGL. The other patients were lost to follow-up
ND data not available

7.3.2 Prediction of relapse in Crohn's disease

Analysis was only performed on those patients who relapsed or remained in steroid free remission. This kept the data 'clean' since other outcomes may have indicated that intestinal inflammation is prevented by steroid or immunosuppressive therapy. Patients requiring surgery have also been excluded from the risk analysis on the basis that surgery could be required for reasons other than intractable inflammation e.g., stricturing.

7.3.2.1 WGLF C3 as a predictor of relapse

The median WGLF C3 value for all patients with inactive Crohn's disease was 0.8 μg/ml (range 0.3-3.7 μg/ml). From this, a high C3 group was designated as WGLF C3 of >0.8 μg/ml and a low C3 group with WGLF C3 <0.9 μg/ml. The upper limit of the reference range for WGLF C3 has been set as 0.9 μg/ml. The frequency of relapse in the two groups was tested by chi square test:

TABLE 7.8 WGLF C3 in patients with different Crohn's disease outcome

patient group	relapsed	steroid free remission	χ^2
WGLF C3 <0.9 μg/ml	8	5	4.0, P = 0.047
WGLF C3 ≥ 0.9 μg/ml	2	8	

There was a significant difference in the frequency of patients that relapsed in each group ($P<0.05$). As a greater proportion of relapses occurred in the low WGLF C3 group, this indicated that relapse of patients with inactive CD was more likely when the WGLF C3 was low. This appears directly opposite to the expected result, especially in light of the earlier finding where WGLF C3 was raised in patients with active disease compared with inactive disease.

This finding has been supported by comparison of survival curves for time to relapse in both high and low WGLF C3 groups by Kaplan-Meier analysis. From the graph (Fig 7.1) it was clear that those patients with inactive CD who had low WGLF C3 relapsed more quickly. Comparing this difference by log rank test gave a small significant difference between the survival curves with a 4.2% chance of being random. Although this was a relatively weak link, it would indicate that low WGLF C3 was a predictor of relapse in patients with inactive Crohn's disease.

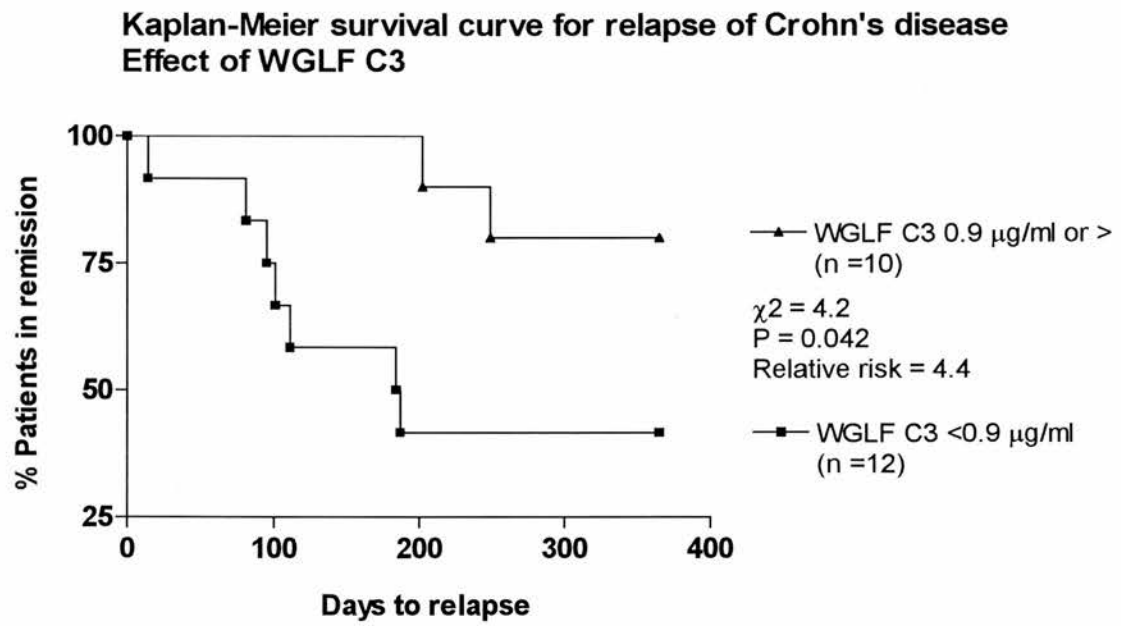

Fig 7.1: Crohn's disease outcome in patients stratified according to WGLF C3

The final point on the graph at time to relapse = 365 days represents patients who remained in remission throughout the follow up period.

In the patients who relapsed, there was a significant, inverse correlation (Pearson, r = 0.52 P <0.05) between WGLF C3 and time to relapse.

Sensitivity and specificity

The sensitivity of WGLF to predict relapse = % of true positives

TP = true positive (number that relapse after low WGLF C3)

FN = false negatives (relapse after raised WGLF C3)

sensitivity = {TP/(TP+FN)} x 100

= {8/(8+2)} x 100 = 80%

specificity = % of true negatives

TN = remain in remission after raised WGLF C3

FP = remain in remission after low WGLF C3

specificity = {TN/(TN+FP)} x 100

= {9/(9+4)} x 100 = 69%

Positive predictive value of low WGLF C3 to predict relapse = % of patients with low C3 who do relapse

positive predictive value = {TP/(TP+FP)}

= {9/(9+2)} = 67%

To assess whether low C3 was a selective predictor of relapse in CD, a relative operating characteristics curve was required. Patients were divided into those who relapsed against those remaining in remission from all possible WGLF C3 cut-off values from the two data sets (0.3-3.7 μg/ml). Sensitivity was given by the number of patients relapsing with C3 equal or below the cut-off divided by total relapses. Specificity = patients remaining in remission with C3 above the cut-off value divided by total in remission. The false positive rate = (1-specificity) and this was plotted on the x axis v sensitivity on the y for all cut-off values selected. A diagonal line from zero sensitivity to 100 % specitivity and sensitivity gave the situation for a test with random characteristics where it is equally likely to get a false as a true positive result. Any test with a curve above that line had some ability to give true positive results.

At a C3 concentration of 0.9 μg/ml the line was at it greatest distance above the random line, demonstrating that the WGLF C3 test is at it's most sensitive to predict relapse at the cut-off value selected for Kaplan-Meier analysis.

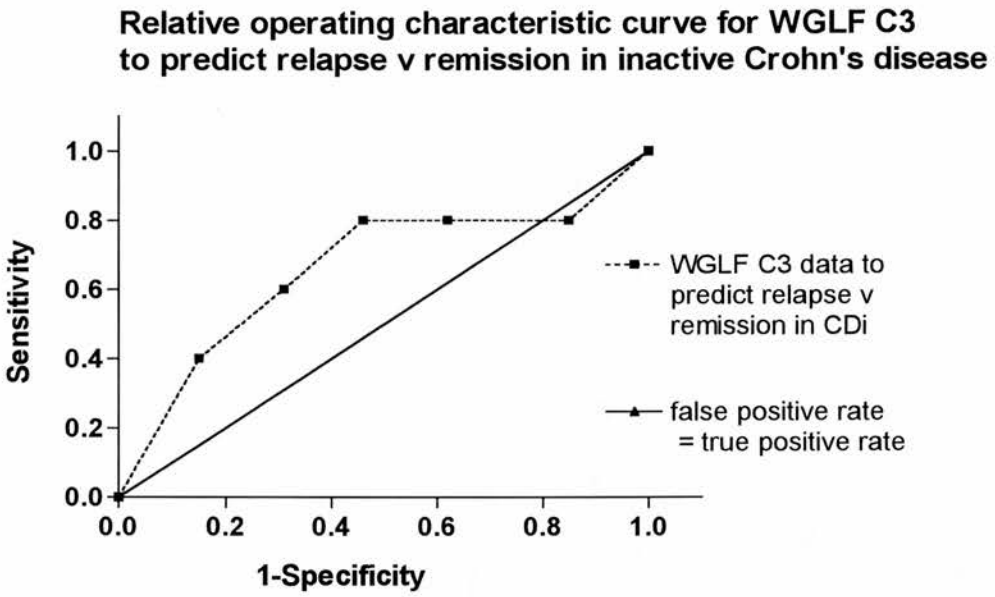

Fig 7.2: relative operating characteristic curve for WGLF C3 to predict relapse as opposed to remission in patients with inactive Crohn's disease

The area under the curve was 0.66 (calculated using Graphpad Prism). This means that 66 % of the time, WGLF C3 values below a cutoff value predicts relapse as opposed to remission at one year follow-up of patients with inactive Crohn's disease. This is a weak predictive parameter that is unlikely to be useful in the clinical setting.

7.3.2.2 Relationship between IL-1β and relapse

The median WGLF IL-1β value for all patients with inactive CD was 16 pg/ml (range 2-535 pg/ml). Patients with a clinical outcome including relapse or steroid free remission were assigned to a low WGLF IL-1β (up to 16 pg/ml) or a high IL-1β group.

From a population of non-inflammatory gastro-intestinal hospital patients and healthy volunteers, the upper 95th percentile of WGLF IL-1β was 12 pg/ml. This is assigned as the upper limit of 'normal' . In this group of patients with inctive CD, I have selected a value of 17 μg/ml to give approximately equal numbers of patients in the IL-1β high and low groups. Testing the frequency of relapse in the IL-1β high and low groups gave

TABLE 7.9 WGLF IL-1β in patients with different Crohn's disease outcome

patient group	**relapsed**	**steroid free remission**	χ^2
WGLF IL-1β < 17 pg/ml	2	8	4.7, P =0.030
WGLF IL-1β ≥ 17 pg/ml	7	4	

There was a significantly greater proportion of patients relapsing in the group who had high IL-1β. Kaplan-Meier survival analysis shows that those patients with high WGLF IL-1β relapsed more quickly. When the difference between the curves was analyzed by log rank test, a significant difference in the relapse rate is seen with only a low chance (1.8%) of this being random. Consequently, high WGLF IL-1β does predict relapse in Crohn's disease, confirming previous results (Arnott et al, 1998). The relative risk was similar to that observed previously with a larger patient group of 70 patients. .

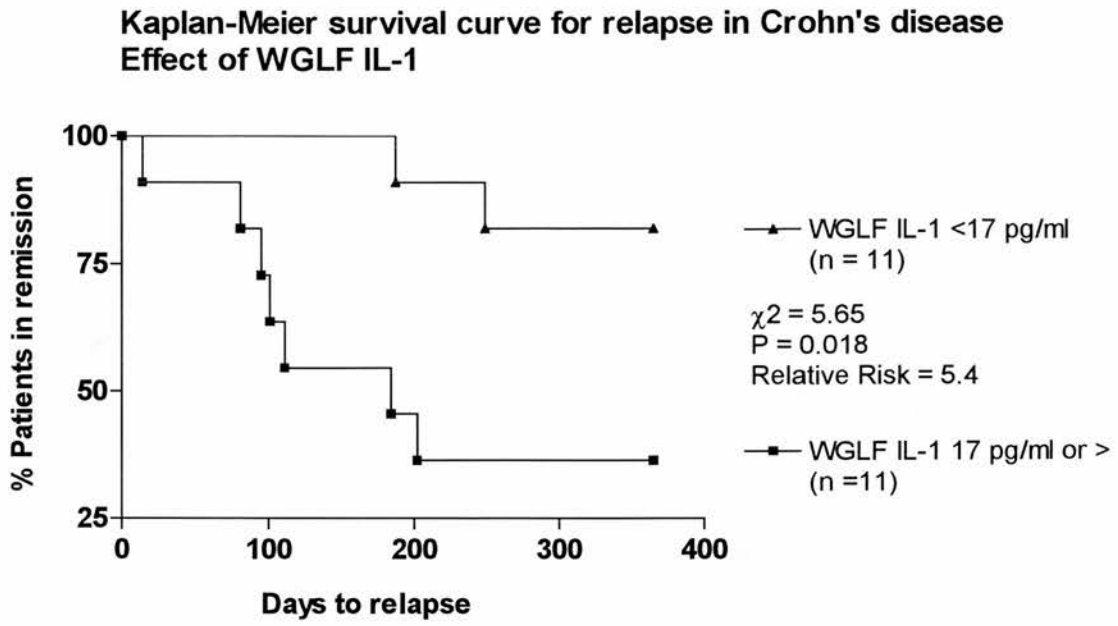

Fig 7.3: Crohn's disease outcome in patients stratified according to WGLF IL-1

Sensitivity and specificity

A cut-off value of 16 pg/ml for WGLF IL-1β was used to give equal numbers in the IL-1β high and low groups. This prevents a possible bias caused by a chance occurrence of a high proportion of a particular outcome in a small group for IL-1β. Previously, a cut-off of 12 pg/ml (from the reference range) had been used. Using this new cut-off, a sensitivity of 70% for WGLF IL-1β to predict relapse as opposed to remission was observed. The specificity for raised IL-1β to predict relapse was 75% and the positive predictive value was 64%. Compared to previous results the sensitivity is lower but the specificity and positive predictive values are higher. There was one patient with a borderline WGLF IL-1β of 16 pg/ml who relapsed whereas 3/13 patients in remission had WGLF IL-1β of between 12 and 16 pg/ml.

7.3.3 WGLF C3 in patients who remained in steroid dependent remission

Patients requiring steroids thoughout the follow-up period to remain in remission show similar concentrations of WGLF C3 to those who remained in steroid free remission.

TABLE 7.10 WGLF C3 in steroid dependent v independent remission of CD

patient group	WGLF C3 μg/ml, median and (range)	P (Mann Whitney U test)
Steroid dependent	1.1 (0.3 -3.2)	0.85
Steroid free remission	1.0 (0.3 -2.1)	

Those patients with inactive CD who had raised WGLF C3 were more likely to remain in remission. These patients had normal concentrations of WGLF albumin, and so plasma leakage was unlikely to be the source of this complement. This may indicate local biosynthesis of C3.

7.3.4 Correlation between WGLF C3 and IL-1β in follow-up patients with inactive Crohn's disease

To investigate whether there was a link between WGLF C3 and IL-1β, correlation (Pearson) was used.

1) In patients who relapsed (n =10) r = -0.116

2) In patients who had remained in remission (n =13) r = 0.14

3) In all inactive Crohn's disease patients (n = 42) r = 0.023

There was no association between WGLF C3 and IL-1β in patients with inactive Crohn's disease.

7.3.5 Comparison of CDAI values

TABLE 7.11 Comparison of CDAI values in CD patients prior to one year follow-up. Patients assigned to groups of disease outcome

	CDAI (median and range)	**P value (compare to remission)**
Remission (11)	85 (25-136)	
Steroid depenndent Remission (7)	106 (41-149)	0.18
Relapse (8)	89 (60-137)	0.41

No significant difference in initial CDAI value was seen in the different CD disease outcome groups. Disease outcome was therefore unlikely to have been related to their disease activity at the start.

7.3.6 Do the high relapse risk group show increased loss of plasma proteins to the gut ?

Increased intestinal permeability is a risk factor for relapse in CD. In active intestinal inflammation there was greater leakage of plasma proteins to the gut, reflecting increased intestinal permeability.

The association between raised WGLF IL-1β and loss of plasma proteins (IgG and albumin) was assessed in patients with inactive CD who either relapsed or remained in steroid free remission.

TABLE 7.12 WGLF IgG in patients with different Crohn's disease outcome

Patient group	**WGLF IgG μg/ml (median and range)**	**P (Mann-Whitney U)**
Relapsed (n =10)	2 (1-8)	0.93
Remission (n =13)	3 (1-8)	

TABLE 7.13 WGLF albumin in patients with different CD outcome

Patient group	WGLF albumin μg/ml (median and range)	P
Relapsed (n =10)	7 (2-21)	0.8
Remission (n =13)	8 (1-21)	

This might indicate that a pre-clinical inflammatory state exists in the intestine of some patients with inactive CD. This was characterized by increased production of pro-inflammatory cytokines and increased epithelial permeability but not increased vascular permeability as seen in active disease. Increased loss of C3 to the gut indicates that the epithelium must be permeable to large macromolecules (mw 180 Kd). If albumin (mw 69 Kd) was able to cross the vascular endothelium, then it was likely that it could cross the intestinal epithelium. The finding of very low WGLF albumin suggests that the vascular endothelium was not permeable to macromolecules.

7.4 Discussion

This study has shown that the risk of relapse in patients with inactive Crohn's disease is linked to the concentration of inflammatory proteins in WGLF. My results for WGLF IL-1β confirm previous findings in this laboratory (Dr I.Arnott, MD, 1998). This demonstrated that the cohort of CD patients studied did not behave in an unexpected manner.

I have used a slightly higher WGLF IL-1β, cut-off value than did Dr Ian Arnott with his cohort. This give a relative risk for the patients with raised IL-1β to relapse of 5.4 as compared with 5.8 from Dr Arnott's work. This finding supports the hypothesis that remission in Crohn's disease is a condition of low grade inflammation and that relapse occurs when mucosal concentrations of e.g. inflammatory cytokines reach a threshold level.

This finding may be useful in the context of a clinical trial where it may be important to have a group of patients 'at risk' of relapse in order that the efficacy of a treatment to maintain remission can be tested.

Because the patients studied were chosen on the basis of having had a whole gut lavage, it is likely that these patients had some symptoms to warrant endoscopic investigation and so might have been at a greater risk of relapsing than the Crohn's disease population as a whole.

It was expected that raised WGLF C3 would be a risk factor for relapse because loss of C3 from plasma would reflect intestinal inflammation as might intestinal synthesis of C3. The finding that low WGLF C3 predicts poor outcome in CD was paradoxical. As no correlation was seen between WGLF IL-1β and C3, the role of C3 in relapse does not appear to be simple. Of ten patients with inactive CD who relapsed during twelve month follow up, two who had raised WGLF C3 might have been predicted to relapse on the basis of raised WGLF IL-1β. Two patients with normal WGLF IL-1β relapsed but had low WGLF C3 that might have predicted this. WGLF IL-1β and C3 therefore appear to work separately as risk factors for relapse in Crohn's disease.

It is possible that failure to detect complement in WGLF does not mean that complement is absent from the intestinal mucosa. It may be that complement is activated and deposited on host intestinal cells in some patients. This would be more likely to result in inflammatory damage to the intestine than complement which simply oozed out of the mucosa. It is possible that complement plays a protective role e.g., by clearing immune complexes and dying cells. In this scenario, detection of complement in WGLF might indicate sufficient complement within the intestinal mucosa to perform this role. Some patients with low WGLF might have rapid complement consumption in the intestine. In these patients low WGLF complement might indicate intestinal inflammation and increased risk of relapse.

A local inflammatory reaction within the intestine might result in recruitment of inflammatory cells such as neutrophils. When these cells have performed their function of removing antigen, they die by apoptosis (programmed cell death). These cells must be removed to prevent their deposition in the tissues, with potential release of powerful enzymes and reactive oxygen species.

Evidence for a role of complement in apoptotic cells comes from the finding that expression of decay activating factor is reduced on apoptotic neutrophils in culture (Jones and Morgan, 1995). It had been shown that apoptotic Jurkat cells (a T-cell line) were able to bind C3b when incubated with serum (Matsui et al, 1994). Deposition of iC3b upon apoptotic cells improved their uptake by macrophages (Takizawa et al, 1996). Complement receptor 3 on macrophages has been shown to be responsible for uptake of complement coated apototic cells by using the gene for this receptor in cell transfection studies (Mevorach et al, 1998). A shortage of complement (or an excess of apoptotic cells) may result in failure to remove apoptotic cells from the intestine with release of cytotoxic agents and resultant tissue damage.

The possibility that WGLF C3 was low in some patients because of consumption associated with removal of apoptotic cells may be investigated by counting apoptotic cells in biopsy specimens and staining of deposited C3 by immunohistochemistry. In active IBD, leakage of plasma C3 into the intestinal mucosa might supply more than is required for opsonization of apoptotic cells. The excess may be detected in WGLF.

Macrophages and intestinal epithelial cells have been shown to be capable of producing C3. IL-1 appears to be a key cytokine involved in upregulating production of complement by these cells (Moon et al, 1997), increasing both mRNA expression and protein secretion. Later studies with transfected cells or inhibitors of NF-Kβ have shown that nuclear factor Kβ is involved in the upregulation of C3 synthesis by IL-1. Macrophages produce IL-1 in response to phagocytosing foreign material. As IL-1 stimulates C3 synthesis, this might assist in removal of antigen. However, several inflammatory cascades are linked to activation of NF-Kβ.

Intestinal production of IL-1 therefore has the potential to be inflammatory. It would be of interest to look at macrophage expression of NF-Kβ in intestinal biopsy specimens from patients with IBD and also to detect mRNA for C3 in these specimens. There may well be a correlation between WGLF IL-1 and C3 mRNA expression.

One explanation for low WGLF C3 predicting relapse in CD could be that these patients allready require immunosuppresents at the start of the follow-up period. These agents might inhibit production of complement in the intestinal mucosa, giving rise to a low WGLF C3. The requirement for immunosuppresents indicates that abnormal inflammatory events are underway within the intestinal mucosa and that an unknown event could trigger excerbation of this with subsequent relapse.

WGLF C3 is unlikely to be used as a tool for predicting relapse in CD because low C3 is found in most subjects without IBD. The study has ignored those patients who had surgery during follow-up and yet this is a more important outcome to predict than relapse. However, the findings have pointed to other mechanisms for the inflammatory process in CD, with potential for further study.

These studies have demonstrated that complement is detectable in WGLF and this may be a useful technique with which to study the immunological mechanisms of intestinal disease. Whilst these findings may have some clinical relevance, this would require a more rigorous clinical study. As a Ph.D student with a biochemistry background, this was beyond my remit.

CHAPTER EIGHT

Conclusions

8.1 Detection of proteins in intestinal secretions

Whole gut lavage has been shown to contain proteins secreted from the intestinal wall. The procedure is used routinely in preparation of intestinal investigation of some patients and so lends itself to comparison of protein secretion in different patient groups. With the WGL technique, protein secretion into the intestinal lumen has been shown to be at a steady-state over a limited timespan. This may allow comparison of protein secretion with that using other, more invasive methods of intestinal perfusion. However, WGLF contains demonstrable protease activity, which is very likely to influence protein recovery. Unfortunately, previous studies of WGLF proteins have not investigated protein recovery. To validate this work and my own it has been necessary to investigate the structural integrity of WGLF proteins and how this influences their quantification.

It was decided to investigate the structural integrity of immunoglobulins in WGLF because they are present in detectable quantities and are directly relevant to the immune function of the intestine. Using a combination of column chromatography and electrophoresis to separate intact from fragmented WGLF immunoglobulin, it has been shown that IgG and IgA are mostly intact. Antigen specific antibodies are therefore likely to be detected by capture ELISA and furthermore, total immunoglobulins may be accurately quantified by sandwich ELISA using anti heavy chain total immunoglobulins. Incubation of unprocessed WGLF at 37°C results in reduced immunoglobulin detection, my inference from which is that immunoglobulin fragments are not detected by sandwich ELISA.

These results validate WGLF as a source of intact intestinal immunoglobulins, supporting published results for detection of immunoglobulins in WGLF. However, different proteins may show varying degrees of resistance to proteolysis.

Immunoglobulins are relatively resistant because of their tight folded domain structure which protects them from the active site of proteolytic enzymes. Other proteins such as albumin are more rapidly degraded in unprocessed WGLF. These proteins may be underestimated in WGLF, reducing the value of quantitative results. These findings have now lead to validation of protein recovery in WGLF before use of that parameter in clinical studies e.g., with insulin-like growth factor-1, a cytokine involved in intestinal fibrosis in CD (Ghosh et al, 1997).

8.1.1 More recent work with WGLF

More recent work at the GI lab has compared protein secretion in ileostomy effluent (IE) to that in WGLF from the same patients. From the finding that daily protein loss calculated from WGLF was much greater than that calculated from IE it was concluded that the high intraluminal flow during the WGL procedure stimulates protein secretion (Croft and Ferguson, 2000). If this was true, it may invalidate all results with WGLF. However, I believe there is flawed logic in that the output of ileostomy fluid is not constant during the day and so calculation of daily protein secretion from a single specimen is likely to be inaccurate. Secondly, the transit time for the ileostomy effluent may be greater than during the gut lavage procedure. Proteolysis during gut transit is therefore likely to be greater with IE. Protein recovery in IE compared with WGLF was greatest for IgM, a protein which is less resistant to proteolysis than other proteins. This suggests that protein recovery in IE relative to WGLF was dependent on resistance of the protein to proteolysis.

Sami Hoque at the GI unit has used WGLF to demonstrate that intestinal immunity in healthy volunteers from Dhaka, Bangladesh differed from that in subjects from Edinburgh, UK. He showed that WGLF from the Dhaka individuals had higher total IgA, IgA to lipopolysaccharide core antigens of gram negative bacteria and eotaxin, a chemoattractant of eosinophils. Conversely, WGLF from the Edinburgh individuals contained higher levels of antibodies to food proteins.

The hypothesis from this is that where the intestinal immune system needs to keep out high levels of bacteria (from dirty drinking water), IgA antibodies to those bacteria are produced by the intestine.

However, because immune capacity in the intestine is stretched, antibodies to food antigens are not produced to the same degree. One outcome of this is that the strategy for mucosal vaccination e.g., against enteric infections such as rotavirus may need to be adjusted for different regions of the world (Hoque et al, 2000).

Outside the GI lab, there are two quotations for measurement of proteins in WGLF from humans. In the first, radiolabelled chromium-EDTA was included in the ingested fluid as a marker of intestinal permeability. The percentage of the marker appearing in urine was measured. This was correlated with the intestinal excretion of calprotectin, a neutrophil marker which was quantified in WGLF by ELISA. Both intestinal permeability and excretion of calprotectin were higher in patients with IBD, levels were relative to the disease activity in CD. A strong correlation between the two parameters was seen as evidence that increased intestinal permeability might be caused by increased migration of neutrophils (Berstad et al, 2000).

It might alternatively be the case that an increase in intestinal permeability caused by something else results in increased migration of neutrophils to the gut in response to chemotactic stimuli. Neutrophil migration to the gut is an early indication of relapse in IBD. An advantage of using WGL in this type of study is that because of the shortened transit time, the patient has reduced exposure to the radiolabelled marker.

The second paper looked at detection of telomerase activity in patients with colerectal carcinoma. Telomerase is an enzyme which replaces the protective cap at the ends of the chromosomes. In most adult cells this enzyme is not expressed and this cap is shortened at each round of cell division. This limits the number of times that cells can divide. Cancer cells do express telomerase and this allows them to divide repeatedly. Patients with colorectal cancer had detectable WGLF telomerase whereas normal subjects did not. WGLF was used because it has been technically difficult to detect telomerase activity in faeces (Ishibashi et al, 1999).

WGL continues to be used in animal studies of intestinal immunity where problems of patient number or heterogeneity are less troublesome e.g., in a study of J-chain knockout mice, it was shown that protection against cholera toxin was abrogated (Lycke et al, 1999). It may well have continued research/diagnostic use in humans where the analyte of interest shows poor or variable recovery in faeces.

WGL as a method for obtaining intestinal contents for biochemical analysis is inflexible in that it can only be performed on patients who require bowel cleansing prior to further investigation. Despite drinking of Klean-Prep being well tolerated in most subjects, it does cause uncomfortable bloating and distension of the stomach. From personal experience I would not like to undergo gut lavage again if it were just for biochemical tests.

However, in those patients undergoing gut lavage for bowel cleansing, it is relatively easy to collect specimens for analysis. As the material is liquid it is easier to process and proteases inhibitors are added within fifteen minutes of sample collection, reducing further proteolysis. Despite between patient variation in the protease activity of intestinal contents, the similarity in transit time during the gut lavage procedure may reduce biological variation in protein recovery due to proteolysis. It is also possible to assess protein secretion over a short time-period by collection of subsequent liquid stools. This may improve the sensitivity of comparing intestinal protein loss between patients. This comes with the proviso that secretion of proteins into the gut during high flow rate perfusion is unlikely to be the same as under physiological conditions.

In contrast, collection of faeces is simple and non-invasive. However, before it is suitable for biochemical analysis, proteins of interest must be extracted. This may involve breaking the faeces up into a suspension with a buffer, followed by clarification of the supernatant by centrifugation. Protein secretion can be assessed using faeces but this requires collection of all faeces passed over the course of a day. It is difficult to make sure that the patient retains all specimens and to process each specimen as it is supplied. Faecal protein concentrations can be compared between patients but the faecal water content needs to be estimated. The major drawback with faeces is the wide variation in transit time and hence proteolysis during transit.

Despite this, some proteins such as the granulocyte marker calprotectin are resistant to proteolysis. Faecal calprotectin was found to be a useful marker of intestinal inflammation which was predictive of relapse in IBD (Tibble et al, 2000). The gastrointestinl laboratory in Edinburgh have since been using this test.

8.1.1.1 The source of proteins secreted into the gut

It is impossible to identify the secretion source of a particular protein in the GI tract and this is one of the major drawbacks of WGLF. However, since some proteins such as IgG are secreted in increased amounts in patients with intestinal inflammation (active IBD), it is reasonable to assume that those parts of the intestine which are inflamed should be the sites where these proteins are secreted. The sites of intestinal inflammation can be identified using endoscopic or radiological procedures.

8.1.2 Do proteins lost to the gut in patients with intestinal inflammation reflect alterations in mucosal biosynthesis or plasma leakage ?

The WGLF concentrations of IgG and C3 (which might be produced in the intestinal wall) were compared to that of albumin (which is serum derived). To control for the concentration gradient between serum and the intestinal lumen, WGLF proteins were first compared relative to their serum concentrations before comparing this ratio to that for albumin. Because of the relative instability of albumin in the gut, it has been necessary to control for differences in albumin recovery in several ways.

1. Only patients with intestinal inflammation restricted to the colon where examined, thus minimizing the time of exposure of albumin to gut proteases.
2. The rate of intestinal perfusion (drinking polyethylene-glycol perfusate) was strictly controlled to reduce variation in this.
3. Protease activity was measured in unprocessed WGLF. The use of WGLF albumin as a marker of plasma leakage was restricted to those patients without extremes of WGLF protease activity.

The relative coefficient of excretion ratio which compares the intestinal relative to serum concentration for a protein as a ratio compared to the same comparison for albumin, a marker of serum leakage, was developed for study of intestinal protein secretion in healthy individuals (Jonard et al 1994). They used perfusion of an isolated jejunal segment which is likely to reduce proteolysis of secreted proteins as duodenal contents (containing digestive enzymes) were excluded from the perfused segment. Consequently it is likely that their results show greater recovery of albumin in the intestinal perfusate than is the case with WGLF. As the serum component is underestimated in WGLF this bias tend to give RCE values greater than one i.e. greater protein secretion into the gut than is attributable to leakage from serum alone.

In light of this, it was very surprising to find that my results for intestinal loss of IgG in patients with active IBD did not provide evidence for greatly increased mucosal synthesis. In active IBD, the number of IgG producing cells in the intestinal mucosa are increased many fold and so it would be expected that the RCE for IgG would be increased. Two explanations for this finding are 1) in the undamaged gut, movement of serum proteins into the gut lumen is dependent on the size of the protein because of the small size of pores in the vascular endothelium of mucosal blood vessels. In contrast, inflammation in the intestine causes these pores to be opened up and protein movement is then dependent on the concentration gradient between the blood and the intestine. As this concentration gradient is high for IgG (with a serum concentration of 15 g/l) it may be that the quantity of IgG which leaks from serum is in excess of that which can be produced by the intestinal mucosa. Consequently, despite massively increased mucosal synthesis of IgG, the RCE value does not reflect this. 2) There was some difference in the IgG RCE between inactive and active disease and the reason for this not reaching statistical significance may have been a type 2 statistical error where there are not enough observations.

In contrast, the RCE values observed with C3 were raised in patients with CD. WGLF C3 was also raised in active ulcerative colitis but in this case it could not be said that the RCE was definitely raised above that which indicates that intestinal C3 is mostly serum derived. This may indicate greater mucosal synthesis of C3 in CD compared to UC.

As the serum concentration of C3 is much lower than that of IgG it is likely that mucosal synthesis of C3 needs to be raised to a lesser degree than does IgG synthesis in order to register a raised RCE. This pinpoints a flaw in using the RCE ratio to assess the source of WGLF proteins in patients with intestinal inflammation where the mechanism of protein loss to the gut is likely to be different to that in individuals with an intact intestinal barrier.

The finding that some patients with inactive CD have raised WGLF C3 without raised WGLF albumin further supports the hypothesis that C3 is synthesized in the intestinal mucosa. As the molecular weight for albumin is one third of that for C3 this finding would indicate that was produced in the intestinal mucosa, the finding that there is increased epithelial permeability without increased mucosal vascular permeability. Increased permeability of the intestinal epithelium would increase the interaction between the mucosal immune system and luminal antigen, potentially resulting in an intestinal inflammatory response.

This may be an over-simplification because the intestinal mucosa is not a fluid bag bordered by two permeable membranes (the vascular endothelium and the intestinal epithelium). The lamina propria contains cells and extracellular matrix which might restrict diffusion of proteins to the intestinal epithelium. Interaction with cells and the extracellular matrix by some proteins such as C3 and IgG may initiate inflammatory reactions. During inflammation it is likely that macrophage metalloproteinase activity results in a more fluid extracellular matrix.

8.1.3 WGLF C3 as a predictor of relapse in Crohn's disease

The finding that WGLF C3 was slightly raised in patients with inactive CD supports the hypothesis that remission may be a subclinical state of intestinal inflammation.

As WGLF C3 is further increased in active CD, it is possible that C3 contributes to an inflammatory process involved in the switch between low-grade and pathological inflammation. To test this hypothesis, patients with inactive CD were followed up for a year after the whole gut lavage procedure. Disease outcome was compared in patients stratified according to their WGLF C3. Paradoxically it appeared that those patients with low WGLF C3 were at greatest risk of relapse. From the literature, two possible hypotheses could explain this finding.

1. Complement activation and deposition on intestinal tissues may consume C3, reducing the quantity detected in WGLF. A falsely negative WGLF C3 result would therefore be seen in those patients at greatest risk of an escalating inflammation of the intestinal mucosa.
2. C3 may be consumed in a protective role in removal of apoptotic cells by macrophages. Deposition of apoptotic cells in the tissues may release enzymes and other potentially inflammatory agents. It might be worth counting apoptotic cells in mucosal biopsy specimens from patients with IBD e.g, by using Hoescht nuclear staining.

Immunological detection of complement fragments in WGLF gave an incomplete picture as it was shown that WGLF C3 is incapable of fixation. It is not known whether C3 activation has occurred within the intestinal mucosa or, in the gut lumen.

Macrophages have been indicated as a possible source of intestinal C3. More recent work has used insitu hybridization to demonstrate the presence of mRNA for C3 in macrophages of the submucosa in patients with CD (Laufer et al, 2000). These macrophages were CD68 positive which suggests that they are recruited from blood. The crypt epithelium also contained C3 mRNA and this correlated with the presence of ulceration and polymorphonuclear leukocytes in the intestinal lumen. This may indicate that generation of chemotactic fragments of epithelial complement is responsible for chemotaxis of reactive neutrophils.

From this, it is likely that any future study of proteins in WGLF might be used as a pilot study prior to using more sophisticated techniques.

8.2 Animal models of IBD

Study of the immunological mechanisms involved in inflammatory bowel disease by biochemical measurements from patients has two main problems: firstly that it is difficult to know what stage of the inflammatory cascade the analysis is detecting. Unless patients can be strictly monitored following their first episode it may not be possible to pick up early events. In many cases, relapse/exacerbation of the disease is detected. It is unlikely that patients having their first episode of IBD can be efficiently recruited into studies because their diagnosis is not usually made until much later.

Secondly, genetic and environmental differences between patients may influence the severity/ nature of the inflammatory response, making it difficult to make conclusions from detection of particular agents. One way around these problems is to use an animal model of IBD.

IBD is a complex disorder with multiple manifestations. Not surprisingly no single model encompasses all the features of human IBD. The morphology of the intestinal wall and it's immune cells is likely to differ between species. In addition, the variable genetic background/ environment in the human population may contribute to some of the features of IBD which only appear in some patients e.g., fistulae in CD. A single strain of animal may not develop some of these features under any treatment regimen. Nethertheless, the four different types of animal model have contributed to our understanding of some of the pathophysiological features of IBD.

8.2.1 Spontaneous models

Cotton top tamarin

This is one of the best models of colitis as the disease spontaneously develops in monkeys exposed to a normal gut flora. The disease follows a course of relapse/remission which mimics human UC. (Madara et al, 1995)

C3H-HeJBir mouse

This is an inbred strain which is prone to colits (Sundberg et al, 1994) Inflammation is characterized by strong T-cell responses to common bacterial antigens. The cytokine profile is high IFN-γ and low IL-4 i.e. Th1. On transfer of CD4+ T cells to immunodeficient mice, these develop colitis. Restricted V beta usage suggests limited bacterial e.g, involved (Cong et al, 1996). Before transfer show no signs of disease (regulatory cells present) CD45RB transfer- IL-10/TGF-β regulatory cells (Powrie et al, 1996)

8.2.2 Induced models

8.2.2.1 Models involving administration of exogenous agents

Enema

1) TNBS

TNBS (trinitrobenzene sulphonic acid) in combination with ethanol induces colitis characterized by a CD4+ T-cell infiltrate of the colonic mucosa. Pre-treatment with antibodies to CD4 prevents development of the disease. TNBS is a contact sensitizer which, if given orally without ethanol can induce oral tolerance. Use of the TNBS/ethanol enema then gives reduced inflammation characterized by up-regulated mucosal TGF-β and IL-10 and decreased IFN-γ. TGF-β was shown to be responsible for the protective effect by a worsening of disease following blocking antibodies. Suppressor T-cells are believed to produce the anti-inflammatory cytokines IL-10 and TGF-β but these cells may be less effective in inflammatory states were oral tolerance may be abrogated (Neurath et al, 1996).

2) Acetic acid enema

In rats this results in acute inflammation of the colon with transient up-regulation of macrophage cytokines e.g, TNF-α. Messenger RNA for these cytokines returned to normal levels when the intestinal histology also reverted to normal. This model is criticised because of its transient non-chronic nature and there is no evidence for T-cell involvement as evidenced by levels of IL-2, IL-4 or IFN-γ (reviewed by Abreu-Martin and Targan, 1996)

Oral administration

Dextran sodium sulphate (DSS)

When in introduced into the drinking water of rodents this causes an acute colonic injury. On removal of DSS colonic regeneration begins but a chronic colitis type injury forms. This model is therefore useful for studying defects in gut healing in IBD.

Intracolonic injection

Peptidoglycan polysaccharide (PG-PS)

This agent produces a granulomatous enterocolitis chacterized by T-cells. Inflammation did not develop in athymic rats(Sartor et al, 1993). Inflammation could be reduced by treatment with recombinant IL-10. This is also the case in CD.

8.2.2.2 Gene targeting: Knockout or transgenic

IL-2 -/- knockout mice

It is difficult to understand why lack of IL-2 (required for T-cell proliferation) should produce colitis. It is possible that other mechanisms are involved in regulating T-cell responses in inflammation or that regulatory T-cells are down-regulated.

IL-10 -/- knockout mice

This is a model for CD as there is a dominant Th1 type immune response in this disorder. IL-10 usually counter-balances this. There is evidence that up-regulated Th1 mediated immune responses in the gut might be directed against luminal bacteria as there was no colitis under germ free conditions (Kuhn et al, 1993).

TCR α -/- knockout mice

These develop colitis dependent on whether a normal gut flora is present. Inflammation is characterized by T-cells with a restricted clonality, possibly indicating that immune responses are against a common bacterial antigen (Takhashi et al, 1999)

N-Cadherin dominant negative mice

This is a model to investigate the role of the intestinal barrier in IBD. N-Cadherin is an integral part of tight junctions and this is dysfunctional in these mice. The mice develop UC-like inflammation, possibly because of increased exposure of immune cells in the intestinal wall to luminal contents. (Hermiston et al, 1995).

8.2.2.3 Transfer of cells into immunodeficient animals

CD4+CD45RB^{hi} T-cells into SCID or RAG knockout mice

Transfer of naive T-cells into immunocompromised mice results in inflammation characterized by Th1 T-cell responses with production of TNF-α driven by IL-12 or IFN-γ (Fuss et al, 1998). Although memory T-cells also contained some reactive T-cell clones, if memory cells were transferred with naive T-cells, no colitis was seen (Claesson et al, 1999). This suggests that the memory subset contains counter regulatory T-Cells which may produce IL-10 (Assesman et al, 1999) and TGF-beta. In many spontaneous/induced models, hidden environmental or genetic factors must be present for inflammation to occur. Some of gene targeted models require particular bowel flora etc.

Effector mechanisms in models of mucosal inflammation

IL-12/IFN-gamma mediated pathways

Distinct histopathology of inflammation in the animal models is associated with dominant Th1 and Th2 cytokine profiles. With a Th1 pattern transmural inflammation featuring granulomas with few neutrophils is seen, resembling CD. With a Th2 profile inflammation is more superficial featuring an acute inflammatory cell exudate (neutrophils) with mucosal oedema. This resembles UC.
This dichotomy of cytokine profile between Th1 CD and Th2 UC is supported by TNBS induced colitis in Balb/c mice. These mice overproduce both IL-4 and IFN-gamma but, with IL-4 predominant. The histopathology more closely resembles UC (Dohi et al, 1999). IL-12 is the key cytokine in Th1 mediated injury. In the SCID transfer and TNBS colitis (SJL/J strain) model, established colitis could be treated with anti IL-12. In the TNBS model, anti IFN-gamma was not so effective This was possibly because anti IL-12 results in fas-mediated apoptosis of activated Th1 cells whereas anti IFN-gamma does not (Fuss et al, 1999).

8.2.2.4 Similarities of disease in animal models v human IBD

From the 1997 review by Sartor, I have included some tables outlining the features of animal models v IBD

TABLE 8.1 Clinical course of murine and rat inflammatory models vs IBD

Disease feature	Induced	Genetically engineered	Human IBD
Diarrhoea	DSS +, PG-SS -ve	yes	yes
Weight loss	DSS+, PG-SS -ve	yes	CD +, UC -ve
Natural history	Chronic (PG-SS) self-limited (TNBS, DSS)	Most progressive, self limited	Chronic, relapse/ remission
Extra-intestinal	PG-PS +	B27	yes (20%)

Some animal models of IBD have clinical features of human IBD. The major difference is that phases of relapse and remission are not seen in the induced rodent models. Another consideration may be the age of the animals and duration of disease.

TABLE 8.2 Histopathological features of ulcerative colitis vs genetically engineered rodent models

Disease feature	Models	Human UC
decreased goblet cells	yes	yes
Dysplasia, development of adeno-carcinoma	yes	yes
Lamina-propria mononuclear cells	yes	yes
Lamina-propria neutrophils	sometimes	yes
Crypt abscesses	occasional	yes
Mucosal hyperplasia	yes	no

This shows that animal models of IBD can result in histopathological changes to the colon which resemble those in human UC. Which features are seen depends on the model of injury/ strain of rodent used.

TABLE 8.3 Pathological features of Crohn's disease and induced rat models

Disease feature	PG-PS	TNBS	Human CD
Mucosal ulcers	rare	yes	
Transmural inflammation	yes	ulcers	yes
Granulomas	80%	rare	50%
Fibrosis	yes	ulcers	yes
lymphoid aggregates	occasional	rare	yes
Fistulae	no	no	yes

TABLE 8.4 Cytokine profiles in experimental colitis and IBD

Cytokine	Experimental Colitis	CD	UC
IL-1	increase	increase	increase
IL-1 receptor antagonist	increase	increase	increase
IL-6	increase	increase	increase
TNF-α	increase	sometimes increased	not increased
Chemokines	MIP2 increase	IL-8 increased	IL-8 increased
IFN-γ	increase	increase	not increase
IL-4	not increased	not increased	possibly increased
IL-5	not increased	not increased	increased
IL-12	increased	increased	not increased

MIP2 = macrophage inhibitory peptide 2

8.2.2.5 Potential of animal models to improve our understanding of inflammatory mechanisms in IBD

As I have outlined, several animal models of intestinal inflammation do mimic part of the disease spectrum seen in human IBD. The major advantage of using an animal model is that the disease has a better characterized starting point than with human IBD. This may allow detection of early changes with insight into what tips the balance between self-regulated and self-perpetuating intestinal inflammatory events. In human studies, it is the inflammatory sequalae rather than the initial event that is detected. The animal models have demonstrated that many exogenous agents can be involved in producing similar types of inflammation. In human studies where a single cytokine or complement component is detected, the usefulness of this information is likely to be limited. What may be a useful approach for the future is the use of microarray technology to detect a battery of genes which might be up-regulated in IBD. This may be useful for detecting overlap in susceptibility genes between rodent strains and humans. However, I believe that data for gene expression may need to be substantiated by detection of the protein, either in mucosal biopsy or in intestinal secretions such as WGLF.

Appendix: general G.I lab methods

9.1 Handling and storage of laboratory samples

All samples were given an adhesive label with an individual sample number. This number, along with the patient details was stored on a database (Microsoft Access).

9.1.1 Processing of samples

9.1.1.1 Blood

Blood tubes are centrifuged at 2,500 rpm for 10 mins (1,800 x g) and four x 1 ml aliquots of serum or plasma stored in screw capped tubes (Starstedt, FRG) at -70°C

9.1.1.2 Whole gut lavage

The whole gut lavage procedure

Bowel cleansing preparation prior to endoscopic investigation of bowel dysfunction has been adapted so that the PEG-electrolyte fluid is taken at a rate which maintains a gut perfusion. After overnight fasting to reduce gut solids, subjects are encouraged by a nurse to drink 'Klean-Prep' (Norgine) at a rate of 250 ml/15 min. Klean-Prep is made up with water as directed and is cooled to make it more palatable. Usually the patient will defecate and after drinking approximately 4 l of Klean-Prep, large liquid stools are passed. The first of these which is free of solid faecal matter is taken for processing.

Processing of WGLF

Reagents

1. SBTI - Soya bean trypsin inhibitor (protease inhibitor)
   0.1 g/100 ml PBS (BDH). Solid is stored desiccated at -20°C with solution being stored at 4°C.
2. PBS - Phosphate buffered saline pH 7.2.
   two tablets (Sigma) dissolved in 400 ml distilled water. The pH may need adjusting to 7.2 by addition of solid potassium di-hydrogen phosphate or di-potassium hydrogen phosphate. Solution is stored at 4°C.
3. EDTA - 0.3 M disodium ethylene diamine tetracetic acid (BDH) pH 8.0.
   Dissolve (with warming) 11.0 g in 100 ml. Saturated solution is stored at room temperature. EDTA is a chelating agent which binds calcium and magnesium ions, reducing their action as co-factors for protease enzymes.
4. PMSF - 0.3 M Phenylmethylsulphonylfluoride (Sigma). 1.74 g/100 ml ethanol. Store at room temperature. This is a protease inhibitor.
5. Sodium Azide. 2 g/100 ml distilled water. This inhibits bacterial growth.
6. NBCS - Newborn calf serum (Sigma heat-inactivated). This is stored in aliquots at -20°C, with the current batch at 4°C for two months. NBCS is a protein 'decoy' to mop up residual protease activity.

Processing procedure

This is done within 20 min of the patient producing a clear specimen.

1. Two 25 ml universal containers of clear lavage fluid are collected. .
2. If the samples were not very clear, they are centrifuged at 1,800 x g for five min.
3. 10 ml of lavage are filtered through Whatman GF/A 12.5 filter paper, collecting filtrate in a universal tube.
4. Aliquots of unfiltered unprocessed lavage are taken and 30 µl of sodium azide added per 1.5 ml. Unfiltered unprocessed lavage is used for the estimation of haemoglobin and for estimation of protease activity.

5. Both filtered and unfiltered gut lavage fluid are taken for processing. 5 ml of lavage is transferred to a new universal tube and treated as follows:

6.

Add 0.5 ml SBTI, mix (by vortex mixer).

Add 0.28 ml EDTA, mix.

Add 0.12 ml PMSF, mix.

Add 0.06 ml Na Azide, mix.

Leave for 2 min.

Add 0.3 ml NBCS, mix.

7 Processed gut lavage fluid is stored in 250 µl aliquots. Filtered processed WGLF is used for estimation of antibodies and other proteins. Unfiltered processed WGLF is used for the estimation of IL-1, where filtration has been shown to remove it.

8. All samples are stored at -70°C. Once an aliquot has been used for an assay it is disposed of and not re-frozen. Repeated freezing and thawing of WGLF is not recommended and has been shown to be deleterious for the recovery of some proteins.

All of the proteins measured in WGLF have been shown to be stable for at least one year at -70°C.

9.2 Laboratory methods for analysis of selected proteins in WGLF

9.2.1 Albumin

Principle of method

Human albumin reacts with it's specific antibody to quickly form precipitating immune complexes in the presence of polyethylene glycol. If the antibody is present in large excess, these precipitates produce turbidity which is related to the concentration of albumin in the sample. Turbidity of immune complexes is photometrically measured at 340 nm. Standards are used to generate a calibration curve, from which the concentration of albumin in the sample is derived (Brydon et al, 1993).

Equipment

Spectrophotometer

Turbidity is measured using a Pye Unicam PU8640 spectrophotometer, set up as follows:

Set to ABS (absorbance) mode
Set wavelength to 340 nm
Set delay to 0.01 min. This time lapse between filling the sample cell and reading reduces interference from sample frothing.
Set volume to 0.8 ml

Diluter

WGLF is diluted using the Gilson Dilugil V. This is set to take 0.05 ml of sample with 0.95 ml of diluent

Test tubes

Assays are carried out in 2 ml polystyrene tubes (LIP Ltd) 64 x 11 mm

Reagents

All reagents are analar grade and obtained from Merck (BDH) unless otherwise stated. They are stored at 4°C unless otherwise stated.

PEG Reagent

40 g polyethylene glycol 6000 (biochemical grade), 6 g Tris, 2 g Tween 20 (Sigma), and 1 g sodium azide dissolved in 800 ml distilled water, adjusted to pH 7.0 with dilute hydrochloric acid and made up to 1 litre volume with distilled water.

Anti human albumin (sheep)

(SAPU - code S034-205). Store frozen in aliquots of 1.0 ml at -20 C.

Antibody Reagent

Dilute sheep anti human albumin serum 1 in 40 with the PEG reagent on the day of assay.

Standard diluent

9 g sodium chloride, 60 g PEG 3350 and 1 g sodium azide in 1 litre solution distilled water.

Standard serum SPS-01

(Protein Reference Unit, Sheffield). Keep at 4°C.

For each batch the accompanying sheet gives the albumin concentration. Standard is diluted with diluent on the day of assay to give a calibration curve of: 0,10,20,50,100 and 200 μg/ml standards.

Quality control

This is WGLF from a healthy volunteer (blood donor) to which serum from the same volunteer is added to simulate leakage as in inflammatory bowel disease.

Stored in aliquots of 0.5 ml at -70°C.

Analytical procedure

1. Bring PEG and Diluent reagents to room temperature.

2. Blanks:

Dilute 50 µl diluent (B1), standards (SB), and test samples (TB), with 0.95 ml PEG reagent in 2 ml polystyrene tubes, in duplicate.

3. Tests:

Dilute 50 µl diluent (B2), standards (S), and test samples (T), with 0.95 ml Antibody reagent in 2 ml polystyrene tubes, in duplicate.

4. Read: Blanks and Tests after 15 - 20 minutes at 340 nm using the spectrophotometer.

Zero reading with PEG reagent. This requires 3 - 4 aspirations before reading stabilizes. Read corresponding blanks and tests for each sample followed by PEG reagent; this will tend to minimize any errors caused by any baseline drift.

Calculations

1. Calculate B2 - B1 = OD value for 0 µg/ml albumin
   S - SB = OD values for 10 - 200 µg/ml albumin, T - TB = OD values for test samples.
2. Plot a graph of OD 340 nm against standard concentration and read test sample results from this; calculate and report mean values.

Assay performance

Repeat analysis within batch gives a coefficient of variation of 8.5% at a value of 40 µg/ml. The QC value should be within 17% (+/-) of expected value for the run to be acceptable.

Reference range

Reference range based on results from 62 individuals, seven of whom were healthy volunteers and the remainder were patients with non-inflammatory gastrointestinal conditions. Using the upper value encompassing 95 % of all results, the normal range was designated from 0 - 26 ug/ml.

Clinical significance of results

In 102 patients with inflammatory bowel disease (71 Crohn's and 31 ulcerative colitis) with heterogeneous disease activity, 52% of cases show raised WGLF albumin. IgG was raised in 64% of these patients. As it has been shown that raised WGLF IgG > 10 μg/ml correlates with a disease activity score of > 150, these patients have active disease. Albumin is therefore less specific for active disease than is IgG. The sensitivity of the assay is reduced because of the variable recovery of albumin in WGLF due to action of gut proteases.

9.2.2 Alpha-1-antitrypsin in Whole Gut Lavage Fluid

Principle of method

Human alpha-1-antitrypsin (A1AT) reacts with it's specific antibody, quickly forming precipitating immune complexes in the presence of polyethylene glycol. If the antibody is in large excess, these precipitates produce a turbidity which is related to the concentration of albumin in the sample. The turbidity is photometrically measured at the wavelength of 340 nm. Absorbance readings obtained by assaying calibration standards are used to generate a standard curve, from which the concentration of A1AT in the sample is derived (Brydon et al, 1993).

Analytical procedure

This is similar to the method for albumin except that anti human A1AT (goat) is used instead of anti albumin.

Quality control

As for albumin assay. This gives a within batch variation of 10 % and between batch variation of 12%.

9.2.3 Haemoglobin in WGLF

Principle of method

Haemoglobin is converted to fluorescing porphyrins by the removal of iron. Total haemoglobin is determined by reaction with heated oxalic acid/FeSO4 reagent which converts haem to porphyrin without loss of pre-formed porphyrins. A three step purification procedure eliminates other interfering fluorescent materials which may be present.

References: The haemoquant assay (Schwartz et al, 1983), was adapted for WGLF (Brydon and Ferguson, 1992)

Sample storage and stability

UFUP WGLF stored at -70°C are analysed within 2 months.

Instrumentation

Fluorescence is measured using the LS-5B Luminescence Spectrometer. Set the excitation wavelength to 402 nm and emission wavelength to 600 nm. Leave instrument for 5 minutes to stabilize before using. The excitation slit width is set to 15 nm and the emission slit width to 5 nm.

Reading

1. Place blank sample in cell carrier and press the autozero key. The digital display reads zero.
2. Place standard sample in the cell carrier, set value e.g. 1,000 for 100.0 µg/ml and press autoconcentration key. The digital display then reads 1000.
3. Repeat steps 1 and 2 to finalize calibration.
4. Test samples can now be read.

Reagents

All reagents are analar grade and obtained from Merck (BDH) unless otherwise stated. Solvents are stored in a metal cabinet and all reagents stored at room temperature.

Oxalic acid reagent

4.0 g oxalic acid (analar) is made up to 10 ml with distilled water in a stoppered glass tube. This is dissolved by heating in a water bath at 100°C. To this, 0.31 g $FeSO_4$ is added with mixing before returning the tube to 100°C. Then, 0.10 g uric acid and 0.11 g mannitol are added with mixing and the final volume adjusted to 10 ml. The reagent is left at 100°C for 15 minutes before use. The reagent is prepared fresh before use and used as a suspension.

Ethyl acetate/acetic acid 10/1 v/v

Add 50 ml glacial acetic acid to 500 ml ethyl acetate and then mix.

Potassium acetate 3 M

29.4 g of potassium acetate (BDH) is dissolved in 100 ml distilled water.

Potassium acetate (3 M) in 1 M potassium hydroxide

Dissolve 147 g potassium acetate in about 300 ml distilled water. Add 28 g potassium hydroxide, dissolve with stirring and make up to 500 ml with distilled water.

n-Butanol

(Rathburn Chemicals Ltd, Walkerburn).

orthophosphoric acid (2M) /glacial acetic acid 9/1 v/v

Dissolve 68 ml orthophosphoric acid in 300 ml distilled water. When cool, make up to 500 ml with distilled water and add 58 ml glacial acetic acid.

PEG 4000 reagent

Dissolve 60 g PEG 3350, 9 g NaCl, and 0.2 g sodium azide in 1 litre of distilled water. Use to make Drabkins reagent.

Drabkins Reagent: (Sigma cat. no. 525-2)

Each vial contains: 1 g sodium bicarbonate, 0.2 g potassium ferricyanide, and 0.05 g sodium cyanide (NB poison).

Take 1 vial and reconstitute to 1 litre with PEG 4000 reagent.

Cyanomethaemoglobin standard

Dissolve 10 mg haemoglobin (Sigma - H 7379) in about 50 ml Drabkins reagent. Leave at room temperature for 15 minutes. Add 6 g of PEG 3350 and make up to 100 ml with Drabkins reagent. To calculate actual haemoglobin concentration, use a spectrophotometer to read the OD at 540 nm against Drabkins reagent (blank). Molar extinction coefficient (540 nm) = 44.0. The haemoglobin concentration can then be adjusted and sodium azide added to 0.02 % before aliquoting into 0.5 ml Eppendorf tubes and storage at -70°C. This is stable for up to 6 months.

Quality control

Use UFUP WGLF where the patients serum has been tested for HIV and Hepatitis status. Haemoglobin (Sigma - H7379) is added to give a haemoglobin value of 10 μg/ml. Aliquots are stored at -70°C and the QC value should be determined against an established control for several runs.

Analytical procedure

1. WGLF must be frozen at -70°C and then thawed to allow haemolysis of intact red cells. After thawing cells are separated by centrifugation at 1,500 x g for ten mins and the supernatant used for assay.

2. Add 0.4 ml of the oxalic acid reagent suspension to 0.1 ml of WGLF supernatant, quality control, haemoglobin standard, and blank (Klean-Prep) in 10 ml stoppered quickfit tubes. Mix thoroughly and heat at 100°C for 30 minutes in a covered water bath. After this time remove the tube stoppers and allow samples to cool for 2 mins.

3. Add 1 ml of the 3 M potassium acetate reagent, followed by 3 ml ethyl acetate/acetic acid reagent. Vortex mix for 30 seconds. This extracts the porphyrin analytes into the upper organic phase.

4. Transfer 2 ml of the upper organic phase to a 30 ml stoppered quickfit tube and add 0.8 ml butanol and 6 ml of 3 M potassium acetate in 1 M KOH. Mix the tubes for 30 seconds. This removes coproporphyrin and other porphrins not derived from haemoglobin haem (those containing more than 2 carboxyl groups are extracted into the lower alkaline aqueous phase).

5. Transfer 1 ml of the upper organic phase to a 10 ml stoppered quickfit centrifuge tube and add 4 ml phosphoric acid/acetic acid reagent. Mix for 30 s. Remove the top layer which contains chlorophyll. Read the fluorescence of the lower acid extract.

6. See instrumentation section for reading the fluorescence. Tests readings /10 give results in μg/ml.

Reproducibility of assay

The coefficient of variation (between batch reproducibility) of quality control material currently gives a value of 8% at 14 μg/ml. The run is acceptable if the QC value is within 2 SD (16%) of mean value.The method is sensitive to 0.5 μg/ml.

Reference range

Reference values for lavage haemoglobin range from 0 -5 μg/ml (Brydon and Ferguson 1992).

Clinical significance of results

The reference range for WGLF haemoglobin is 0-5 μg/ml which is equivalent to daily blood loss of 1 ml. Values in excess of this indicate pathological GI blood loss.

Interferences and limitations

Other haem containing proteins have the potential to interfere in this assay, e.g. myoglobin in meat or peroxidases in vegetables, although neither of these should cause significant interference in WGLF. WGLF which is contaminated with faecal material should be avoided.

9.2.4 ELISA assays

ELISA = Enzyme linked immunosorbant assay

General procedure

Non competitive ELISA assays are used to measure total or antigen specific Immunoglobulins G, A or M in serum or WGLF. A double antibody sandwich technique is used for quantifying total immunoglobulin, using a purified secondary standard material. An indirect technique is used for estimation of antigen specific immunoglobulins measured against a known high human sample.

Class specific antihuman immunoglobulin or pure antigen is bound in excess to a solid phase and then washed. In these methods, a 96 well ELISA plate is the solid phase, with different plate types have different binding characteristics. The plates are incubated with a protein containing solution to "block" any non specific binding sites. Samples are added to the plates in doubling dilutions covering a wide range of concentrations whilst samples for antigen specific analysis are added at a single predetermined dilution. Samples are incubated and then washed. The starting dilution is predetermined by testing at what dilution the response is modulated by further sample dilution.

Binding takes place between the antihuman globulin and the immunoglobulin being quantified or between the specific antigen and corresponding antibody.
A class specific antihuman antibody conjugated with alkaline phosphatase is added to the plate, incubated and then washed. This conjugated antibody binds to the complex bound to the solid phase. On adding substrate, the amount of product is proportional to the amount of conjugate bound and hence the amount of antibody in the sample.

ELISA reagents

SPS-01

Standard serum for quantifying IgG and IgM. Dept.of Immunology, P O Box 894, Sheffield S5 7YT. Once opened, this is stored for one month at 4°C

Human IgA (colostrum)

Standard material for quantifying IgA in WGLF (Sigma, I-2636). This is reconstituted in distilled water to 210 μg/ml and stored in 110 μl aliquots at -20°C

Coating antibodies

Goat antihuman IgG, Fc specific

Affinity purified (Sigma I 2136)

Use at 1/5,000

Goat antihuman IgM, μ chain specific (Sigma I 2386)

Use at 1/5,000

Goat antihuman IgA, α chain specific (Dako)

Use at 1/600

Conjugated antibodies (alkaline phosphatase)

Goat antihuman IgG (Sigma A 9544)

Use at 1/5,000 for estimation of total IgG

Goat antihuman IgM (Sigma A 3437)

Use at 1/10,000 for estimation of total IgM

Goat antihuman IgA(Sigma A3036)

Use at 1/10,000 for estimation of total IgA.

For detecting antigen specific antibodies, the conjugate antibody used must be optimized for that particular assay. A dilution of 1/1,000 is usually a good starting point.

Other reagents

Sterile water

1 litre sterile water containers (Baxter , Australia) stored at 4°C.

Carbonate-bicarbonate coating buffer

0.05 M, pH 9.6 at 25°C. Dissolve the contents of 10 capsules (Sigma C 3041) in 1 litre sterile water. Mix to dissolve and store at 4°C.

sodium chloride

0.9% sodium chloride in 1 litre sterile containers (Baxter Healthcare Code F7124) is stored at 4°C.

Wash solution

0.9% saline + 0.05% Polyoxyethylene-Sorbitan Monolaurate (Tween 20, Sigma P 1379). Add 500 µl Tween 20 to 1 litre of 0.9% saline. Mix and store at 4°C.

Adult bovine serum

Adult bovine serum (SAPU, S026-220, Law Hospital, Carluke, Lanarkshire, ML8 5ES). Store at -20°C. Before use this should be allowed to reach room temperature before filtering through a 22 µm filter (Millipore).

ELISA diluent

0.9% saline + 0.05% Tween 20 + 1% adult bovine serum.

Add 500 µl Tween 20 and 10 ml filtered adult bovine serum to 1 litre of saline. Mix and store at 4°C.

10% Diethanolamine (DEA) substrate buffer

For 2 litres, mix 200 ml diethanolamine (BDH)

0.20 g magnesium chloride ($MgCl_26H_2O$) and

0.4 g sodium azide (NaN_3) in 1.6 litres of sterile water. The pH is then adjusted to 9.8 with 6 M hydrochloric acid before making up to 2 litres with sterile water. This is stored at 4°C and is stable for 4 months.

p-nitrophenylphosphate (substrate)

5 mg phosphatase substrate tablets, disodium p-nitrophenyl phosphate hexahydrate (Sigma 104-105). Tablets are stored at -20°C.

Substrate mix for alkaline phosphatase

Diethanolamine buffer is allowed to reach room temperature. One substrate tablet is dissolved per 5 ml buffer shortly before required.

On adding substrate to alkaline phosphatase (immobilized on ELISA plate) the following reaction takes place:

p-nitrophenyl phosphate (colourless) → p nitrophenol (yellow)

The colour development takes place within an hour and the optical density at is read on a dedicated ELISA reader (set at 405 nm with a reference filter at 630 nm). A Dynatech system programmed with Revelation software is used. The absorbance for all wells is read when that for the top standard has reached a previously determined value. An OD of one has been used as the spectrophotometer is at maximum sensitivity whilst the reading is high enough to give good separation of positive and negative results.

Instrumentation

Dynatech Multi-Reagent Washer

This is a programmable ELISA plate washer fitted with a 16 way dispense head. This allows simultaneous aspiration from two columns of a 96 well plate, which can be immediately followed by filling of those wells with e.g. wash solution. The wash head then moves to the next two columns until the final column where it moves back to the start. Three wash cycles are usually used.

Waste from the ELISA plates is collected in a trap bottle and this can be decontaminated with e.g. Presept, before disposal via the drains.

Dynatech Microplate Reader

The Dynatech MR5000 microplate reader is linked to a Gateway 2000 computer fitted with Revelation software. This allows either instant absorbance reading or reading at a pre determined optical density reading (e.g. for the top standard). A print out of the optical density readings can be obtained or alternatively the program can be used to fit a curve to the standard points and calculate the test unknown concentrations.

9.2.4.1 Estimation of total IgG in WGLF

Test indication

The concentration of total IgG (and also albumin and α1-antitrypsin) in WGL fluid are raised in patients with active gut inflammation. Total IgG is therefore measured in WGL to assist in the diagnosis of inflammatory bowel disease and in patients with confirmed disease as an alternative/additional measure of the degree of inflammation.

Principle of assay

Total IgG in WGLF is quantified against the protein reference standard (SPS-01) using a double antibody sandwich ELISA technique (Gaspari et al, 1988). This has been validated as a marker of disease activity in IBD (O'Mahoney et al, 1991)

Sample requirement and stability

IgG in untreated lavage material is stable for 2 hours at 37°C. IgG should therefore be intact in WGLF following gut transit. Filtered processed WGLF should be used and IgG has been shown to be stable at -70°C for 3 months.

Standard

SPS-01 is diluted in ELISA diluent to give a concentration of 250 ng/ml.

Quality control

WGLF is taken from a patient suspected of having raised inflammatory parameters and a large volume is stored at -70°C. After the patient gives consent, an aliquot of WGLF is tested for Hepatitis B and HIV infection. If these tests are negative and the WGLF contains suitable antibody levels, the WGLF pool is thawed and processed with protease inhibitors. The sample may be diluted with Klean-Prep to give an IgG concentration at the upper limit of the reference range. Aliquots of this are stored at -70°C.

Analytical procedure

Volumes are 100 µl unless stated

Coat ELISA plate

Dilute IgG coating antisera 1/5,000 in carbonate/bicarbonate coating buffer pH 9.6. Add 100 µl/well to an Immulon 1 flat bottomed plate and incubate the plate overnight at 4°C in a moist box.

Wash plate

ELISA wash with three aspirate/dispense cycles using automatic plate washer. Tap plate dry on paper towel.

Block Plate

Add 250 µl of ELISA diluent/well and leave for a minimum of 1 hour at room temperature..

Prepare samples

Allow WGLF and QC to reach room temperature. Dilute the samples and QC material 1/25 in ELISA diluent (40 µl plus 960 µl ELISA diluent).

Add samples to the plate

Dispel the blocking solution from the plate and blot dry on absorbent paper. Using an 8 channel multipipette, add 100 µl of ELISA diluent to all wells except for B1,B2 and A3-A12. To wells B1,B2 and C1,C2 add 100 µl of the appropriately diluted standard. Mix the standard solution and the diluent in C1,C2 by drawing up and down the pipette tip 3 times. Then, transfer 100 µl from C1,C2 to D1,D2 and so on to produce a series of doubling dilutions. Similarly, after vortex mixing diluted WGLF samples, add 100 µl to duplicate columns (e.g., A3,A4 and B3,B4). Then by mixing the sample and diluent in B3, B4, produce doubling dilutions. Cover plates with a plastic lid and incubate overnight at 4 °C in a moist box.

Conjugate

Dilute the conjugated antisera to 1/5,000 in ELISA diluent. Wash the plates before adding 100 µl of conjugate to each well. Cover the plate with a plastic lid and incubate at 24°C for 3 hours.

Colour development

Prepare alkaline phosphatase substrate (15 ml per plate) 30 mins before required. After washing the plate, add 100 µl of substrate solution to each well. The plate can then be placed in the ELISA reader and the OD monitored. My program read the absorbance at 405 nm against a reference of 630 nm when the OD for the first standard reached 1.0 (after subtracting blank readings).

A standard curve of OD against $\log_{10}$ of concentration gives a sigmoid curve with a linear central portion. A straight line equation can be fitted to this region using linear regression. From this equation, test concentrations are extrapolated. Mean values from the doubling dilution points most parallel to the standard are taken before multiplying by the sample dilution factor.

Assay requirements

The correlation coefficient for the standard curve must be greater than 0.985 with QC values within 2 SD of the expected value. Samples must have 2 or more points parallel to the standard curve. Samples with OD readings greater than that for the top standard are tested again but at a greater dilution of 1/250.

Clinical significance of results

The reference range for WGLF IgG is 0-10 µg/ml. Total IgG is the most sensitive parameter for inflammation in the gut with raised values indicating gut inflammation.

Assay performance

Between batch CV =12.3% at 19.8 µg/ml

9.2.4.2 ELISA for total IgA and IgM

are similar with the following changes:

Total IgA

Coating

Coat Dynatech Immulon 1 plates 100 µl/well with rabbit anti human IgA diluted to 1/600 in carbonate-bicarbonate buffer pH 9.6

Sample preparation

Filtered processed WGLF is diluted 1/100 in ELISA diluent before adding 100 µl/well to the plate. Doubling dilutions are produced as before.

Standard

Sigma human colostrum IgA (I 0633) is diluted in ELISA diluent to 1 µg/ml. This is diluted in doubling dilutions on the plate.

Conjugate antibody

Sigma goat anti human IgA (α chain specific) alkaline phosphatase conjugate is diluted to 1/10,000 in ELISA diluent. 100 µl is added to all wells.

Total IgM

Coating

100 µl/well of anti human IgM (µ chain) at 1/10,000

Samples

WGLF is diluted from 1/25

Standard

SPS-01 is diluted to give 1 µg/ml IgM, with doubling dilutions on the plate.

Conjugate antibody

Use anti IgM alkaline phosphatase conjugate at 1/10,000.

9.2.5 ELISA for measuring antibodies to specific antigens

General assay differences

ELISA plate

Binding of a purified antigen requires a 'stickier' ELISA plate such as the gamma irradiated Dynatech Immulon 2.

Coating

Test the optimum coating concentration of antigen but expect this to fall between 1 and 10 μg/ml

Sample dilution

WGLF diluted from 1:1

Conjugate antibody

There will be fewer antibodies capable of binding to the immobilized antigen than when detecting antibodies of all specificity (total antibodies). The conjugate antibody should be used at a greater concentration e.g. 1/1,000

9.2.5.1 ELISA for detecting antibodies to ovalbumin

All volumes 100 μl unless stated

Coating

Coat Immulon 2 plates at 5 μg/ml with ovalbumin from chicken egg (Sigma) dissolved in coating buffer. Incubate the plate in a moist box for 5 h at 24°C. Wash 3x with ELISA wash (0.05 % Tween in saline) and blot plate on paper tissue..

Blocking

Fill wells with ELISA diluent and leave for 2 h at 24°C

Samples

Dilute serum 1/100 for IgA and IgM, 1/200 for IgG. Lavage should be tested at 1:1.

Standard

A serum sample 96/1548 from a patient with untreated coeliac disease is used. This has been designated as containing 528 arbitrary IgA anti ovalbumin units (by reference to an original standard material). Storage of aliquots of standard material is at -70°C. This is diluted from 1/264 on the plate.

For IgG a starting dilution of 1/675 = 0.4 IgG units

For IgM 1/390 = 1 IgM unit

After adding samples and standards, the plate is incubated overnight at 4°C

Conjugate

Sigma anti human IgA, G or M are used at 1/500. Add 100 µl/well and incubate the plate for 5 h at 24°C.

Colour reaction

After washing the plate, the colour reaction with p-nitrophenyl phosphate is carried out as before. The OD at 405 nm is read when that for the top standard reaches 1.0

Reference range

By drawing a frequency curve of values obtained from 120 hospital patients without coeliac disease and 13 healthy controls, the following 95th percentile limits were obtained:

IgA 30 units

IgG 62 units

IgM no clear cut-off.

Clinical significance

Many people have some serum antibodies to ovalbumin but patients with coeliac disease more frequently have raised antibodies. However, the presence of anti ovalbumin antibodies is not specifically indicative of coeliac disease. No reference ranges have been assigned for WGLF and the clinical significance of these antibodies has not been investigated in detail.

Quality control

Serum from a coeliac patient is used.

Assay performance

This shows a within batch CV of < 10% and a between batch CV of < 15%.

9.2.5.2 ELISA for antibodies to LPS core antigen (EndoCAb)

This is a semi quantitative ELISA to detect antibody to endotoxin developed by Dr Robin Barclay at the blood transfusion service, Edinburgh. From the original method to measure specific IgG in serum (Barclay and Scott, 1987), Samiul Hoque and myself have adapted and optimized the ELISA to measure antibodies in WGLF.

Principle of method

ELISA plates are coated with endotoxin core antigen cocktail (smooth an rough mutant LPS from Eschericia Coli, Klebsiella pneumoniae, Pseudomanas aeruginosa and Salmonella minnesota complexed with polymyxin B. Plates kindly supplied by Dr Robin Barclay, blood transfusion service, Edinburgh.

Reagents (from Sigma unless stated)

Sample buffer (for plate washing and sample dilution)

1 tablet PBS buffer / 200 ml

1 ml Polyoxyethylene Sorbiton Monolaurate (Tween 20)

5 ml 10 % sodium azide

40 g Polyethylene Glycol

10 g endotoxin free bovine serum albumin

To one litre with distilled water

Standard

Human serum with high titre, supplied by Dr Barclay. This has been designated a number of arbitrary units for IgA, IgM and IgG against the median response from a Scottish healthy volunteer population. This is diluted 1/250 for IgG, 1/50 for IgM and 1/25 for IgA.

Samples

Filtered processed WGLF is diluted from 1:1

Assay procedure

(all volumes 100 µl)

Sample addition

The standard and WGLF samples are added to the plate and doubly diluted in duplicate columns. After adding samples and standards, the plate is incubated for 90 mins at 37°C.

Conjugate antibody

The plate is washed 3x with ELISA wash solution to remove unbound material. Conjugate antibody against the appropriate antibody class is then added at a dilution of 1/1,000. The plate is then incubated for three hours at 24°C.

Colour development

Unbound conjugate is washed free and substrate (p-nitophenol phosphate 1 mg/ml in 10% Diethanolamine buffer pH 9.8) added. The optical density at 405 nm is read when that for the top standard reaches one.

Calculations

A plot of units on the x axis against OD on the y gives a sigmoidal curve with a linear region usually over a range of four doubling dilution points. A linear regression equation is fitted to those points and the test concentrations calculated from this (Graphpad Prism).

Precision

The coefficient of variation within run was < 4% and between run < 12%.

9.2.6 Other non-ELISA methods

9.2.6.1 Total protein assay

Biorad total protein estimation

This method is a commercial adaptation of the Bradford dye binding procedure. A colour change which can be detected spectrophotometrically results from proteins binding Coomassie blue dye in acidic solution.

The dye binds to aromatic and basic amino acids, especially arginine. The method is useful for quantifying the concentration of proteins with MW greater than 5,000.

A standard for the assay needs to be carefully chosen to reflect the nature of the protein, as dye binding may vary for different proteins. For WGLF, the commercial serum standard, SPS-01 was chosen as this is likely to contain some proteins found in WGLF.

Procedure

Essentially as per Biorad instructions. The optical density of the protein/dye mix was read at 595 nm after 30 mins reaction.

The quantity of reagents used were scaled down for economy, 20 µl of sample + 1 ml of dye instead of 100 µl of sample + 5 ml of dye.

I have found that saturation of dye binding usually occurred with protein concentrations greater than 1 mg/ml. This gave a standard curve which was linear at low protein concentrations but, which flattened off at high concentrations. Some samples were diluted prior to testing so that the response was within the linear range obtained for the standards.

9.2.6.2 Protease activity in WGLF

Gaspari et al (1988) have quantified the activity of protease enzymes in gut lavage fluid, qualifying the requirement for the addition of protease inhibitors prior to storage. They used Azocoll which is an insoluble, ground collagen which is conjugated by a peptide type link to a bright-red azodye. Protease enzymes release the dye into solution, the absorbance of which can then be quantified spectrophotometrically. Under optimal conditions the quantity of dye released is proportional to the protease activity. A method adapted from Gaspari has previously been set up in this laboratory.

Method (Gordon Brydon Ph.D. University of Edinburgh, 1996)

- Make a suspension of Azocoll (Sigma) 10 mg/ml in PBS 0.01 M pH 7.2
- To 2 ml polypropylene test tubes add:
  50 µl of unfiltered unprocessed WGLF or 50 µl of PBS as a blank
- Add 50µl of Azocoll suspension and vortex mix.
- Incubate tubes for 15 min in a waterbath at 37°C
- Plunge the tubes into ice cold water and add 1.5 ml of cold PBS per tube to stop the enzyme reaction.
- Centrifuge the tubes at 1,800 x g for 5 mins to separate any dye which has been released into the supernatant
- Transfer the supernatant using a Pasteur pipette to fresh test tubes
- Read the optical density at 530 nm against the blank test of PBS.

I found that this assay showed poor sensitivity, possibly due to differences in batches of Azocoll.

Optimizing the Azocoll reaction

a) Finding the optimum ratio of Azocoll to WGLF

UFUP WGLF (50 µl) was dispensed into several test tubes and to these, between 50 and 400 µl of Azocoll suspension (10 mg/ml in PBS) was added. The enzyme reaction was carried out for 15 min at 37°C before stopping the reaction. After separating the supernatant, the OD 530 nm was read against a blank reaction tube of PBS + Azocoll.

b) Testing the linearity of the enzyme reaction

To allow comparison of enzyme activity in different samples, it is necessary to compare how much product has been formed within a defined time period. Choosing that time period is a balance between the needs for assay sensitivity and the possibility that at longer times, substrate may be limiting and so the amount of product formed might not truly reflect the enzyme activity. One way of doing this is to look at the linearity of the amount of product formed with time.

If at a point when sensitivity is acceptable, the amount of product continues to increase in a linear manner with time then it is likely that even if testing a sample with greater enzyme activity, it is unlikely that at that timepoint that the amount of substrate would be limiting.

UFUP WGLF (50 µl) was dispensed into several test tubes and to these, 450 µl of Azocoll suspension added. The reaction was proceeded at 37°C with duplicate tubes being removed at two minute intervals. The reaction was stopped in these tubes which were then stored at 4°C until all tubes had been stopped. All tubes were then centrifuged and the OD of the supernatants read. The optimum conditions were then used for all subsequent studies.

Within batch variability of assay

WGLF (UFUP) was assayed twenty times in the same run to test the within batch variability. Between batch variation was tested with ten samples that had been included in more than one run.

Protease activity in filtered unprocessed (FUP)WGLF

Filtered unprocessed WGLF was more frequently available than unfiltered unprocessed WGLF freezer archives. If protease activity could be reliably assayed in FUP WGLF, this would allow a comprehensive retrospective study of albumin recovery in relation to WGLF protease activity. Protease activity was assayed in both UFUP and FUP WGLF.

a) Optimizing the concentration of Azocoll for estimating protease activity

The amount of dye released (OD) increases with increasing amount of substrate added. The plateau in this curve indicates that maximum dye release can be obtained by adding 450 µl of Azocoll to 50 µl of UFUP WGLF.

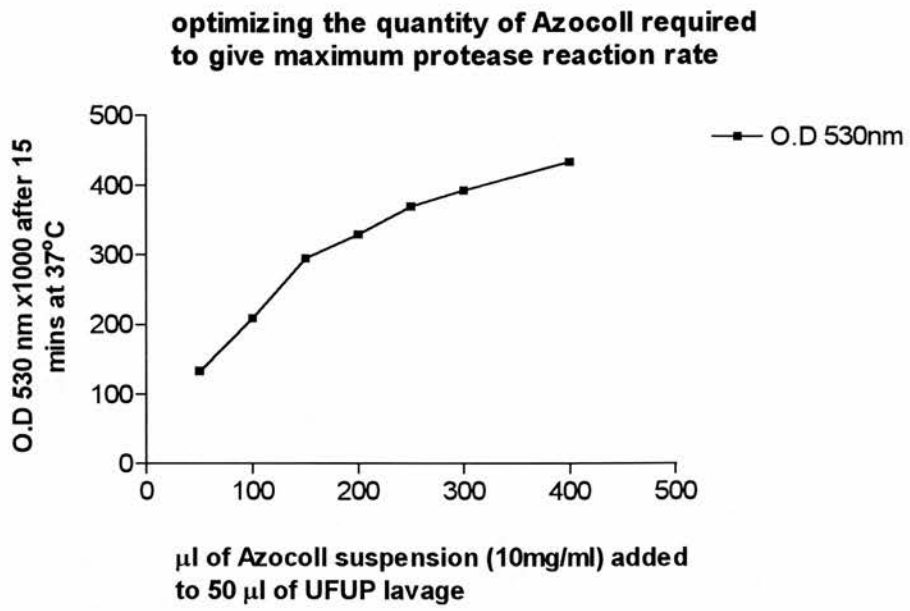

Fig 9.1: Optimizing the quantity of Azocoll required to detect WGLF protease activity

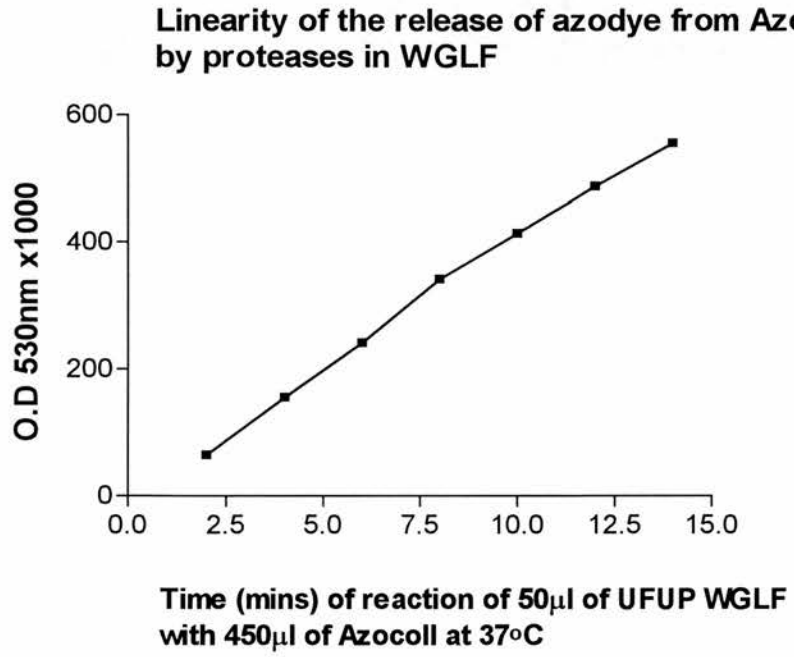

Fig 9.2: Optimizing the Azocoll reaction time in protease assay

As the reaction gives a linear response beyond 10 mins, ten minutes was chosen as the assay reaction time.

9.3 T-Gel appendices

9.3.1 SDS-Polyacrylamide gel electrophoresis; equipment:

a) Vertical slab electrophoresis tank (Batheseda Research Laboratories, USA)

b) Glass electrophoresis plates

c) Plastic spacers (1.5 mm thick) and gel comb

d) Bulldog clips

e) Buchner flask with side arm

f) Vacuum pump

g) Gel loading pipette tips

h) Powerpack capable of delivering constant current (Shandon, UK)

Reagents:

From Sigma unless stated

a) Stock 50% acrylamide:

1.25% N,N -methylene bisacrylamide and 48.75% acrylamide (filtered through Whatman no 1 paper and stored at 4°C in a brown bottle.

NB This is a neurotoxin and so gloves are worn.

b) 10% Sodium lauryl sulphate (SDS) in distilled water

store at rt. and warm to dissolve before use.

c) Stacking gel buffer, 1 M Tris HCl pH 6.8

121.14 g Tris (Tris (hydroxymethyl) methylamine, BDH, UK)

d) Resolving gel buffer, 1 M Tris HCl pH 8.8

e) Stock 10 fold electrophoresis buffer 1.92 M glycine , 0.25 M Tris pH 8.3 (BDH). Before use this is diluted ten fold with distilled water and SDS included to a final concentration of 0.1%

f) TEMED (fresh)

g) Reducing sample buffer:

6% SDS, 6% (v/v) 2-mercaptoethanol, 40% (w/v) sucrose and 0.02% bromophenol blue in 0.125 M Tris HCl pH 6.8.

h) Sigma wide range MW markers (range of MW from 6,500 to 205,000)

i) Ammonium persulphate 1.5% in distilled water (made fresh)

Protein staining reagents

a) Coomassie brilliant blue stain (BDH, UK)

0.2% w/v solution in 1:1:0.2 mixture of methanol : d.H_2O : glacial acetic acid. Filter through Whatman n°1 paper before use.

b) Destaining solution

0.5:8.5:1 mixture of methanol : d.H_2O : glacial acetic acid

Assembling the gel mould

Glass electrophoresis plates for a 18 x 13 cm cast were cleaned with distilled water followed by 70% ethanol. To the front and rear surfaces of the gel spacers, silicone grease was smeared. Of the two glass plates, the front plate has a rectangular cut out at the top, to allow access to the cast gel. The rear plate was lain flat and the greased spacers placed in position on top of it, forming a continuous barrier along the base and side edges. The front plate was then placed on top and the unit sealed with bulldog clips.

Gel formation

A resolving gel of 12.5% w/v acrylamide was chosen because of the small pore size which enables separation of low molecular weight proteins and also because it's rigidity makes it easier to handle. For this size of gel, 40 ml of resolving gel mix is required.

Resolving gel

1. 50% stock acrylamide (10 ml)
2. 1M Tris-HCl pH 8.8 (15 ml)
3. d.H_2O. (13.7 ml)

mix in a Buchner flask and then degas by vacuum for twenty minutes. The following were then added:

4. 10% SDS (0.4 ml)
5. ammonium persulphate (0.9 ml).

The contents were mixed gently and polymerization started by addition of:

6. TEMED (30 µl)

Gel solution was drawn up in a syringe and slowly dispensed into the gel mould along the edge of a side spacer, introducing as little air as possible. Air bubbles were displaced by tapping the outside of the glass with a spatula. The gel surface was overlain with 200 µl of 0.01% SDS (to stop air from getting in as this inhibits polymerization). With the mould positioned vertically, the gel was allowed to set for one hour at rt. Before adding the stacking gel, the surface of the resolving gel was washed with three washes of SDS (2 ml), removing this by capillary action with a filter paper.

Stacking gel

For the usual stacking gel protocol, 5% of acrylamide is used. However, 7.5% acrylamide was used because this sets more readily and forms neater sample wells.

1. 50% stock acrylamide (1.5 ml)
2. 1M Tris-HCl pH 6.8 (1.25 ml)
3. d.H_2O. (6.65 ml)

were mixed in a Buchner flask before degassing. The following were then added:

4. 10% SDS (0.1 ml)
5 ammonium persulphate (0.5 ml)

This was mixed by gentle swirling and polymerization initiated by addition of:

6. TEMED (20 µl)

The liquid gel was decanted onto the surface of the resolving gel and the gel comb inserted into the top of the gel, without introducing air bubbles. The top of the gel was overlain with 0.01% SDS and the gel allowed to set for one hour at rt.

9.3.1.1 Preparing the electrophoresis assembly

When the stacking gel had set, the well comb was removed and unpolymerized acrylamide rinsed from the wells with three washes of electrode buffer (5 ml) The wells were left full of electrode buffer. The cast gel was fitted into a vertical electrophoresis tank (Batheseda Research Laboratories, USA) with the exposed side of the stacking gel against the upper buffer reservoir (cathode). Adhesive rubber strips smeared with silicon grease help to give a water tight seal between the glass plates and the upper reservoir, preventing buffer from that reservoir leaking to the lower (anode) reservoir and so breaking the electrical circuit. Bulldog clips were used to hold the electrophoresis plates firmly in position.

Upper and lower reservoirs of the electrophoresis tank were filled with electrode buffer (0.192 M glycine, 0.025 M Tris pH 8.3 containing 0.1% SDS, ensuring that the wire electrodes and the gel sample wells were covered.
Air bubbles sitting between the base of the resolving gel and the anode buffer were displaced by means of electrode buffer squirted through a Pasteur pipette. This allows an even electric field across the base of the gel.

9.3.2 Western Blotting

Equipment

a) Milliblot SDE transfer system

b) Consort E741 Power Supply

c) Whatman 3MM chromatography paper (UK)

d) Millipore 0.45μm Nitrocellulose NCHAHY grade (UK)

e) Plastic incubation boxes with lids

Reagents

a) Methanol (Analar, BDH, UK)

b) Anode buffer 1:

0.3 M Tris, 10% Methanol pH 10.4 (adjusted with 1 M HCl)

c) Anode buffer 2:

0.025 M Tris, 10% Methanol pH 10.4

d) Cathode buffer:

0.025 M Tris, 0.04 M glycine, 20% Methanol pH 9.4

e) Ponceau S stain

diluted 1/10 in d.H_2O

Assembling the blotting apparatus

Millipore semidry transfer apparatus was assembled as per manufacturers instructions, in brief: The Mylar insulating matrix ensures that current can only flow through the gel sandwich. This was placed over the edges of the lower (anode) electrode.

Six pieces of 3MM filter paper and a piece of nitrocellulose were cut to the size of the gel. The gel was first pre-soaked in anode buffer two to prevent it from swelling during the transfer. One piece of chromatography paper was soaked in anode buffer 1 and then drained to remove the excess. A spot of this buffer was place in the centre of the carbon anode and the chromatography paper placed on top of the this. Air bubbles under the sheet were removed by smoothing the surface with a glass rod. Two pieces of chromatography paper were soaked in anode buffer 2, before placing these on top of the first sheet as before.

Nitrocellulose was first activated by soaking in water before soaking in anode buffer two. A corner was cut off the nitrocellulose to match with the electrophoresis direction marker on the gel. This was then placed on top of the chromatography paper stack. The gel was then placed in position on top of the nitrocellulose and air bubbles between the gel and the membrane removed by smoothing the gel surface with a gloved finger wetted with anode buffer 2.

Three pieces of 3MM paper were soaked in cathode buffer before placing on top of the gel. All handling was done wearing clean gloves to prevent proteins from skin from contaminating the nitrocellulose. The cathode was then placed on top of the gel sandwich and the retaining nuts tightened by hand.

Gel staining with Coomassie brilliant blue

Resolving gel was placed plastic box and flooded with Coommassie blue reagent. The gel was incubated overnight (with rocking) at room temperature. To remove the background staining, the stain was decanted, and destain solution added. The gel was incubated for 2 h before decanting destain and adding fresh. This produced good contrast between the stained protein bands and the gel background. Destain solution was then removed and the gel stored in a sealed plastic box containing 7% glacial acetic acid preservative.

9.4 Computer packages used in production of this thesis

This thesis has been written using Micosoft Word version 6

Data has been stored on Microsoft Excel version 5 spreadsheets

Statistics have been calculated using Minitab for Windows version 10

Graphs have been produced using Graphpad Prism version 1.03

References were stored using Reference Manager for Windows version 6

Patient data was stored on a central laboratory database in Microsoft Access v 5

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LOW WHOLE GUT LAVAGE COMPLEMENT C3 PREDICTS POOR OUTCOME IN INACTIVE CROHN'S DISEASE

Kenneth A. Humphreys, Ian D.R. Arnott, Subrata Ghosh

Gastrointestinal Unit, University department of medical sciences, Western General Hospital, Edinburgh

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INTRODUCTION

Relapse in Crohn's disease

- Clinical relapse may result from biochemical/immunological changes within the intestinal mucosa
- Detection of substances in intestinal secretions may reflect early relapse before clinical signs
- Biochemical predictors of relapse may contribute to our knowledge of the inflammatory mechanism
- High concentrations of pro-inflammatory cytokines IL-1 and IL-8 define patients at high risk of relapse

Complement C3 in Crohn's disease

- Evidence for complement activation in inflamed intestine
- Loss of C3 to the gut increased in active disease
- Macrophages synthesize more C3 in response to IL-1

AIMS

- To quantify C3 in intestinal secretions from patients with inactive Crohn's disease
- To test whether this is related to disease outcome at 1 yr. follow-up

PATIENTS AND METHODS

Patients with inactive Crohn's disease (n =43)

TABLE 1

Male:Female	22:21
Age	54 (20-80)
Disease duration (months)	95 (1-374)
Resections	1 (0-4)

brackets indicate median (range)

- Disease assessed as inactive by WGLF IgG <10 μg/ml
- In most cases CDAI <150

The whole gut lavage procedure

- Bowel cleansing with PEG provides source of intestinal secretions
- Patients drink PEG at a rate of 250 ml/15 min. until clear effluent is passed per-rectum
- Previous studies have demonstrated that when clear fluid is passed, a gut perfusion is established
  with steady state loss of substances to the gut
- Clear WGLF is processed with protease inhibitors before storage at -70^{o}C

Analytical methods

- Total IgG in WGLF assayed by in-house sandwich ELISA
  WGLF IgG correlates well with CDAI >150
- New competition ELISA to quantify C3(c)
  Reference range for WGLF C3 (43 non-inflammatory intestinal disease patients)

0.3-0.8 μg/ml.

Limit of detection 0.3 μg/ml

Clinical outcome

Patients were followed-up for one year after gut lavage

and catagorised as follows:

TABLE 2

Relapse	A change in symptoms requiring oral steroid therapy
Remission	No change in symptoms
Steroid dependent	Requiring oral steroids at follow-up and 9/12 months
Immunosupressive	Require Azothioprine or 6-Mercaptopurine
Resection	Refractory disease requiring surgery

RESULTS

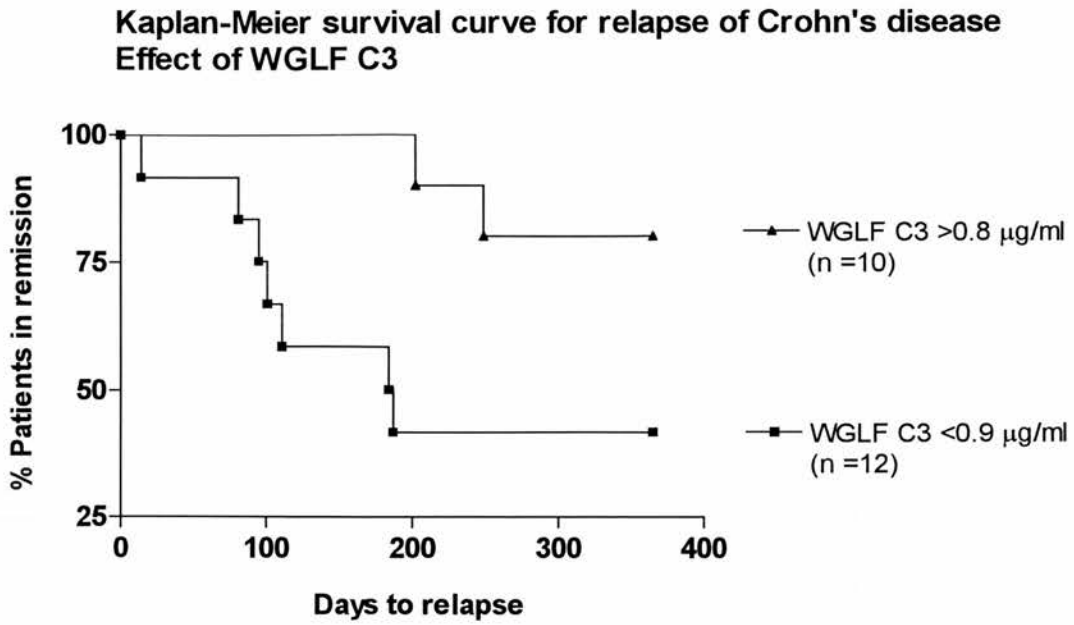
TABLE 3

Outcome classified by WGLF C3

	Relapse	Remission	Steroids	Immunosup	Resection
C3 <0.9 μg/ml	2	8	5	1	3
C3 >0.8 μg/ml	8	5	2	2	3

figures denote number of patients (excluding 4 lost to follow-up)
Immunosup = taking immunosuppresents

- A greater proportion of patients who relapsed had normal levels of WGLF C3 compared with steroid free remission group (P =0.07 χ^2 test)
- Patients relapsing at follow-up as opposed to those remaining in steroid free remission can be analysed on the basis of WGLF C3 using Kaplan-Meier survival analysis (one relapse patient with low C3 omitted as time to relapse not known)
- Curves were compared by the log rank test. The result of this: χ^2 4.2, P= 0.042 is that for patients with inactive Crohn's disease, normal levels of WGLF C3 give a relative risk of relapse of 4.4



- This finding was the exact opposite to that expected i.e. that as WGLF C3 is raised in active Crohn's disease, slight increases in WGLF C3 might indicate early stages of mucosal inflammation.

Possible explanations

1) Those patients who relapse might show increased mucosal C3 but, if this was consumed (tissue deposition) it may be undetected in WGLF. Mucosal C3 consumption may be involved in the inflammatory process.

Previous studies show that complement deposition in the intestine of patients with Crohn's disease is related to the degree of inflammation. Patients with inactive disease should all show low level complement deposition.

- In my study, detection of greater WGLF C3 in active disease does not indicate a reduction in mucosal C3 consumption, it may simply reflect increased leakage of plasma proteins.
- It would be of interest to compare mucosal mRNA for C3 in those patients who relapsed and those remaining in remission

2) Complement could play a protective role in the regulation of mucosal immune responses. This may be especially important in Crohn's disease where there is an imbalance towards inflammatory responses.

- Studies have shown that complement may facilitate uptake of apoptotic cells by macrophages. If this occurs in the gut, it may potentially reduce dangerous tissue deposition of apoptotic immune cells.

The role of complement in removal of apoptotic cells from the intestinal mucosa presents a new area of potential study.
This demonstrates how an unexpected scientific finding can open new avenues of investigation.